THE EFFECTS OF LEUKOTRIENE B₄ ON
OSTEOCLAST FORMATION AND OSTEOCLASTIC
BONE RESORPTION AND THE ROLE OF
OSTEOBLASTIC CELLS IN THESE PROCESSES

by

Victoria Tai

A thesis submitted in conformity with the requirements
for the Degree of Master of Science,
Graduate Department of Pharmacology,
University of Toronto.

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The Effects of Leukotriene B4 on Osteoclast Formation and Osteoclastic Bone Resorption and the Role of Osteoblastic Cells in these Processes.

Victoria Tai, MSc, 1997
Department of Pharmacology, University of Toronto

ABSTRACT

Leukotriene B4 is a lipoxygenase metabolite of arachidonic acid with well-characterized effects on leukocytes and has also been implicated in bone loss associated with inflammatory conditions. Previous experiments by Gallwitz et al. (1993) have shown that LTB₄ stimulated resorptive activity of osteoclasts isolated from osteoclastomas. In the present investigation we have evaluated the role of LTB₄ with respect to formation of osteoclasts and osteoclast precursors and resorptive activity of differentiated osteoclasts in osteoclast-containing cell populations derived from newborn rabbit long bones. Osteoclasts were cultured on devitalized bone slices in the absence or presence of LTB₄. LTB₄ (10⁴ M) stimulated the formation of TRAP+ve multinucleated cells and increased the total area resorbed after 48 hours of culture. To distinguish between direct and indirect effects of LTB₄ on osteoclastic activity and/or formation, we used isolated rabbit osteoclasts cultured with or without added osteoblast-like cells (UMR 106-01 cells). A 24 hour treatment with LTB₄ (10⁴ M) produced no effect in osteoclast cultures alone. However in 24 hour co-cultures of osteoclasts and UMR 106-01 cells, LTB₄ (10⁶ M) stimulated formation of osteoclasts and increased total area resorbed. Furthermore, stimulation of resorption also occurred when UMR 106-01 cells were pre-cultured with LTB₄ for 24 hours and subsequently co-cultured with osteoclasts for 48 hours in the absence of LTB₄. The LTB₄ - mediated stimulatory effects were blocked by a specific LTB₄ receptor antagonist, LY 292 728. Our findings suggest that LTB₄ is a potent stimulator of resorption and induces increased formation of osteoclasts. Our data also suggests that the effects of LTB₄ on osteoclasts are mediated through an effect on osteoblast-like cells in the osteoclast-containing cell populations.
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<tr>
<td>alpha minimal essential</td>
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<tr>
<td>antibiotics</td>
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<td>bone morphogenetic protein</td>
<td>BMP</td>
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<td>calcitonin</td>
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<td>calcitonin receptor</td>
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<tr>
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<td>standard error of the mean</td>
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tartrate resistant acid phosphatase positive

transforming growth factor beta

tumour necrosis factor alpha
CHAPTER 1

GENERAL INTRODUCTION
1.1 INTRODUCTION: BONE AND BONE CELLS

Bone is a specialized connective tissue consisting of a mineralized organic matrix and bone cells. Bone matrix consists of about 65% bone mineral, mainly in the form of hydroxyapatite, which when combined with 35% type I collagen, provides the mechanical strength of the skeleton. The matrix and bone cells form an unique architecture adapted to provide mechanical support and an environment for haematopoiesis. In addition to these roles, bone also serves as the major reservoir for calcium homeostasis.

In order to fulfil these functions, bone is metabolically active such that the coupled processes of formation and resorption continue throughout life. The cells responsible for bone resorption are the osteoclasts, the cells responsible for bone formation are the osteoblasts. Normal bone development and remodelling depend upon intricate temporal interactions between precursor cells, mature bone cells, extracellular matrix molecules and signals generated by systemic and local factors. Bone formation (during development) and remodelling (throughout life) are dependant upon factors that regulate the number and activity of both bone forming osteoblasts and bone resorbing osteoclasts. Bone formation occurs in two stages, matrix formation and mineralization. Active osteoblasts produce an organic bone matrix which consists predominantly of type I collagen (90%) with the remaining 10% comprised of several proteins and proteoglycans such as osteocalcin, osteonectin, osteopontin and bone sialoprotein (Fisher, 1985). This matrix mineralizes through osteoblastic-mediated processes.

There are two kinds of bone: cortical bone (the outer, denser envelope of most bones) which plays a major role in support function and trabecular or cancellous bone which is metabolically more active and forms the supporting network within bone.
1.2 OSTEOBLASTS

Osteoblasts are cells responsible for bone formation. They develop from osteoprogenitor cells which are of mesenchymal origin. When osteoblasts mature, they can differentiate into either lining cells, found along trabeculae and endosteal surfaces, or osteocytes (see Martin, 1994 for review). Characteristics of the osteoblast phenotype include positive staining for alkaline phosphatase (Rodan & Rodan, 1983), production of type 1 collagen and production of bone matrix proteins. Throughout the various stages of differentiation, osteoblasts may only express a proportion of all the phenotypic features that they are capable of expressing (Heersche et al., 1993).

There are many experimental systems available for the investigation of osteoblasts in culture: organ cultures of bone, primary bone cells or established clonal cell lines derived from normal bone (Ng et al., 1988) and cell lines isolated from bone tumours (Majeska et al., 1980, Partridge et al., 1981). The UMR 106-01 cell line that we have used in some of the investigations described here is derived from an osteosarcoma induced by $^{32}$P-orthophosphate injections into Sprague Dawley rats (Partridge et al., 1980).

1.2.1 Osteoblast-Osteoclast Interaction

Chambers et al. (1989) have shown that many stimulators of bone resorption have direct effects on osteoblasts but not osteoclasts. Additionally Chambers has argued that since the osteoclasts were mostly derived from a "wandering cell" it seemed logical that their activation in bone should be directed by "authentic" bone cells and he proposed that osteoblasts are the cells responsible for this. Moreover, osteoblasts may direct the determination of the location of osteoclast resorption. The initiation of bone resorption starts when the osteoclasts gain access to the bone matrix. The bone matrix is covered mostly by lining cells which make the surface
unavailable for osteoclastic activity. For bone resorption to occur, retraction of the osteoblasts from the bone surface is necessary (Rodan & Martin, 1981, Ferrier et al., 1994). Time lapse videomicroscopy showed that osteoclasts travel to the bone surface to resorb bone through invasive behaviour by displacing the lining osteoblasts from their initial position on the bone matrix (Veseley, 1992, Ferrier et al., 1994).

Harris & Heaney (1969) were the first investigators to find a strong correlation between the skeletal-wide level of resorption and formation. They termed this skeletal coupling. Since then it has been recognized that once resorption occurs, osteoblasts respond by making more bone matrix. It is essential for physiological integrity that a balance exist between resorption and formation. Resorbing bone produces a factor which influences the rate of osteoblast activity (Howard et al., 1981) which might be a possible mechanism for the coupling of bone formation and resorption. Howard et al. (1982, 1981) and Drivdahl et al. (1981) have provided evidence that the regulation of bone mass is mediated by locally released bone formation mediators. Additionally the discovery that osteoblasts deposit growth factors (BMPs and TGF-beta) within bone matrix (Urist & Strate., 1971; ten-Dijke et al., 1990, Mundy & Bonewald, 1990) support the concept that osteoclastic resorption releases factors that may stimulate the differentiation of osteoblasts.

1.3 OSTEOCLASTS

Osteoclasts are defined as mononuclear or multinucleated cells that resorb bone and stain positive for tartrate-resistant acid phosphatase (TRAP). Osteoclast progenitors are of hemopoietic origin, and they are recruited from hemopoietic tissues such as bone marrow and circulating blood (reviewed in Baron et al., 1993). Osteoclast progenitors then proliferate and differentiate into mononuclear preosteoclasts and fuse with each other to form multinucleated osteoclasts.
Osteoclasts exist in many shapes and sizes. Distinctive morphological features of osteoclasts include nuclei with prominent nucleoli, a strongly basophilic and vacuolated cytoplasm, highly developed perinuclear Golgi complexes, numerous mitochondria and abundant free ribosomes, and lysosomes (Holtrop, 1991). One of the most striking features of osteoclasts is their abundant mitochondria: osteoclasts contain more mitochondria per unit of cytoplasmic volume than any other cell in the body (Holtrop, 1991). The sheer numbers of this organelle in osteoclasts indicate these cells function at a high level of aerobic metabolism.

The morphological hallmark of active osteoclasts is the ruffled border, a uniquely polarized region consisting of extensive membrane foldings (Scott and Pease, 1956). The ruffled border is always circumscribed by an organelle-free area, the clear zone, which contains numerous contractile/attachment filaments arranged in association with specific integrins (Holtrop and King, 1977; Lakkakorpi et al., 1989). It is likely that this specialized cytoplasmic region limits the acid environment necessary for matrix degradation by sealing off the area under the ruffled border from the extracellular space (Schenk et al., 1967; Lakkakorpi & Vaananen, 1991). The ruffled border is a series of cytoplasmic extensions which are often finger-like, but may also be folded sheets. The ruffled border provides an enormous surface area for the exchange of materials between the cell and extracellular space. Holtrop and colleagues, using serial section analysis, showed that the ruffled border size varies according to cell activity. They found that parathyroid hormone and 1,25 dihydroxyvitamin D3, known stimulators of bone resorption, stimulate an increase in osteoclast ruffled border area in sections of rat bone. Inactive cells have little or no ruffled border (Fukushima et al., 1991). Osteoclasts have been shown to vacillate between active and inactive states (Heersche and Kamehisa, 1988 and Lakkakorpi and Vaananen, 1991). Acidification of the resorption space involves the activity of the vacuolar proton ATPase, which is abundant in the ruffled border. Additional enzymes are required for maintaining the
acid-base balance of the cell in the presence of this massive translocation of protons across the brush border membrane.

To resorb bone, newly formed or quiescent osteoclasts must be activated by factors in the local environment either in response to systemic signals such as 1,25 dihydroxyvitamin D3 or parathyroid hormone (PTH) or local factors including matrix components, cytokines, or growth factors produced by cells in the microenvironment (Rodan and Martin, 1981).

The complex and dynamic patterns of resorption that enable morphogenesis and restructuring are likely to be achieved with instructions from local mesenchymal cells. A major component of this control includes regulation of osteoclast formation from local precursors, and ultimately from hemopoietic stem cells (Chambers et al., 1989).

1.3.1 Origin of Osteoclasts

Osteoclasts have been established to be of hemopoietic origin. This conclusion was reached mainly from in vivo experiments carried out in late 1970s. These included parabiosis experiments (Gothlin & Ericsson, 1972), chick-quail chimera (Kahn & Simmons, 1975) and the pioneering work of Walker and colleagues (1973) with restoration of bone resorption in osteopetrosis by transplanting normal marrow cells or spleen cells (see Osdoby et al., 1993 for review). The experiments of Walker made it virtually certain that osteoclasts were indeed cells from the hemopoietic lineage. He transplanted spleen cells or bone marrow cells from normal mice into irradiated osteopetrotic littermates and found that this resulted in the formation of normal osteoclasts. More recent experiments provide additional evidence that osteoclasts are derived from the hemopoietic stem cell. Scheven et al. (1992) co-cultured purified bone marrow fractions with living osteoclast-free embryonic mouse long bone rudiments and found that the fraction containing hemopoietic stem cells gave rise to osteoclasts. Alvarez et al. (1991) and
Hagenaars et al. (1989) have shown that production of osteoclasts resulted from marrow, blood and splenic macrophage precursor cells. Peritoneal macrophages and cells from the HL60 line of myelo-monocytic precursors are also capable of forming multinucleated bone resorbing osteoclast like cells in the presence of 1-25 dihydroxyvitamin D3 (Bar-Shavit et al., 1983).

1.3.2 Isolated Cell Systems

After the hemopoietic origin of osteoclasts was established, several in vitro model systems were developed to study osteoclasts. Determining the mechanisms by which the bone-forming osteoblasts and bone-resorbing osteoclasts achieve their coordinated activity has been and is a challenging problem for investigators. Given the complex, interactive and heterogeneous nature of the bone environment, the use of isolated cell populations has provided an effective approach for dissecting the individual events involved. There are great benefits in working with purified isolated osteoclasts and likewise there are limitations imposed by removal of osteoclasts and their precursors from the interactions with neighbouring cells and local and systemic regulation (see Cielinski & Marks, 1994 for review). When isolated osteoclasts are cultured on devitalized slices of mineralized cortical bone, bone resorption can be studied without the influence of the cell layer normally covering bone surfaces in vivo and without the added complication of the presence of a nonmineralized layer of osteoid and or an unmineralizable connective tissue component (Chambers, et al., 1984, Boyde et al., 1984). Thus the major advantage of dealing with isolated purified cell populations is the ability to attribute a specific response to the cell under study.

Osteoclasts represent a functionally and morphologically heterogeneous cell population. Parameters such as variation in size, age, stage of differentiation, nuclear content and physiological states all affect bone resorbing activity (Kaneshisa & Heersche, 1988). Osteoclasts
represent a low percentage of the population in comparison with the other cell types present in bone. For this reason, initial attempts to isolate and purify sufficient numbers of viable cells were difficult. Nelson & Bauer (1977) designed the first successful approach using unit gravity sedimentation. Since then, osteoclasts have been isolated from bones using mechanical curetting, sequential incubations with trypsin and collagenase and calcitonin to effect cell release (Chamber & Magnus, 1982; Hefly & Stern 1982; Osdoby et al., 1982). Apart from yield and identification, the issues of greatest concern are cell purity, viability and species. The procedure of Zambonin-Zallone & Teti (1991) using repeated sedimentation allowed purity to be increased, but both yield and viability were compromised. In 1985, Oursler and co-workers reported using a low calcium diet with chick hatchlings to increase osteoclast yield. Sufficient numbers of osteoclasts were produced to enable screening of monoclonal antibodies raised against isolated osteoclasts. Oursler et al. proceeded to develop methods for selectively isolating viable osteoclasts via the development of monoclonal antibodies coupled to small magnetic beads. Although the calcium deficient "mode" of avian osteoclasts isolation is useful to obtain high yields of osteoclasts, there are still uncertainties related to the lack of calcitonin receptors in avian osteoclasts (Nicholson et al., 1987). Calcitonin is a prime regulator of mammalian osteoclast activity and the absence of these receptors in chicken osteoclasts raises doubt that these cells are appropriate models for mammalian osteoclasts.

1.3.3 Co-culture systems, Osteoblastic Cells and the Generation of Osteoclasts

Using a co-culture system, Burger et al. (1991) demonstrated that living bone cells are required for osteoclast development (review Suda et al., 1992). Takahashi et al.(1988) and Taylor et al.(1993) noted that in mouse bone marrow cultures, TRAP+ve osteoclast-like multinucleated cells were formed mainly near colonies of alkaline phosphatase-positive cells.
Takahashi et al. (1988) found that co-cultures of primary mouse calvarial osteoblast-like cell populations with mouse spleen cells in the presence of 1,25 dihydroxyvitamin D3 lead to formation of multiple TRAP+ve mononuclear and multinucleated cells. No TRAP +ve cells were formed in the absence of the vitamin (Takahashi, 1988). Furthermore, the stromal cell lines MC3T3-G2/PA6 and ST2 could be substituted for primary osteoblastic stromal cells in inducing osteoclast development in cocultures with spleen cells (Udagawa et al., 1989).

1.4 SYSTEMIC FACTORS

All factors that stimulate or inhibit bone resorption must act by changing either the activity or the number of resorbing osteoclasts. Major systemic regulators of osteoclast activity and osteoclast numbers are parathyroid hormone (PTH), calcitonin (CT), 1,25 dihydroxyvitamin D3 and steroid hormones.

1.4.1 Parathyroid Hormone

Parathyroid hormone participates in calcium homeostasis by increasing plasma calcium levels through its action on the bone and kidneys. In bone, PTH releases calcium stores by stimulating bone resorption, while in the kidneys PTH acts to increase renal tubular reabsorption of calcium and excretion of phosphate. PTH also enhances the production of 1,25 dihydroxyvitamin D3 thus stimulating intestinal calcium absorption (Garabedian et al., 1974). In vitro, PTH action has been considered to be indirectly mediated through receptors on osteoblastic cells. Enriched populations of osteoclasts do not respond to PTH unless osteoblasts are added (Chambers et al., 1986; de Vernejoul, et al., 1988; McSheehy et al., 1986). Osteoblasts and their precursors in bone (Silve et al., 1982; Rouleau, et al., 1988) have been shown to express PTH receptors. PTH can promote fusion of marrow cells, leading to the formation of multinucleated...
cells with an osteoclast phenotype (Teti et al., 1988). PTH action in bone is mediated through cyclic AMP and/or phosphatidyl inositol related receptor-mediated pathways (Abou-Samra et al., 1989).

1.4.2 1,25 Di-Hydroxyvitamin D₃

1,25 di-hydroxyvitamin D₃ is generally considered to be the most important metabolite of vitamin D with respect to regulation of bone metabolism. It increases plasma calcium levels by increasing intestinal absorption of calcium and phosphorus, as well as mobilizing these minerals from bone. Receptors for 1,25 di-hydroxyvitamin D₃ are present in virtually every cell type studied, including osteoblasts and their precursors (Chen et al., 1982) but such receptors have not been demonstrated in osteoclasts (Merke et al., 1986). The major effect of 1,25 di-hydroxyvitamin D₃ on bone in culture is an increase in osteoclastic activity (Holtrop et al., 1979) without an increase in osteoclast number. No direct effect of 1,25 di-hydroxyvitamin D₃ on bone resorption has been found, but in co-cultures of osteoblast-like cells and osteoclast containing cell populations, 1,25 di-hydroxyvitamin D₃ stimulates resorption (McSheehy & Chambers, 1987).

Osteoclast formation from marrow cultures increases with the addition of 1,25 di-hydroxyvitamin D₃. Prostaglandin production appears to be necessary for osteoclast formation. 1,25 di-hydroxyvitamin D₃ in addition to increasing formation of osteoclasts, also stimulates PGE₂ production in hemopoietic cultures. Furthermore this effect is blocked by indomethacin addition (Fuller & Chambers, 1987; Shinar et al., 1990). A role for PG in osteoclast formation is consistent with evidence from Tashjian et al. (1987) who found that in long term organ culture experiments PG production mediated increased bone resorption induced by EGF, PDGF, TGF alpha and beta, TNF and IL-1. More recently, the stimulatory effect of 1,25 di-hydroxyvitamin D₃ on osteoclast formation was shown to be mediated by members of the 5-lipoxygenase family.
The effects of 1,25 di-hydroxyvitamin D₃ were blocked by specific blockers of the 5-LO pathway (Flynn et al., 1995).

1.4.3 Calcitonin

Calcitonin directly inhibits activity of osteoclasts: decreases the area and motility of the cells and inhibits osteoclastic bone resorption (Chambers & Magnus, 1982). Calcitonin receptors (CTR) were first identified on isolated rat osteoclasts by Nicholson et al. (1986) and expression of calcitonin receptors is now considered one of the most reliable markers of the osteoclast phenotype (Hattersley & Chambers 1989). Binding of calcitonin to osteoclasts via their calcitonin receptors results in an increase in cAMP production (Chambers et al., 1984, Nicholson et al., 1986). However calcitonin does not induce cAMP production in avian osteoclasts and specific binding for calcitonin is hardly detected on avian osteoclasts (Nicholson et al., 1986). The lack of demonstrable calcitonin receptors on avian osteoclasts should be noted when chick osteoclasts are used to investigate osteoclast functions. Osteoclasts over time recover, or exhibit the capacity to "escape", the inhibitory effects of calcitonin (Kaneshisa, 1989). This escape phenomenon shows heterogeneity with regard to responsiveness of isolated osteoclasts to CT in terms of cytoplasmic motility and resorptive activity, between species and with various culture conditions (Heersche et al., 1992). Additionally, CT receptors are downregulated after exposure to CT and there is significant species variability in CTR expression (Obie et al., 1979)

1.4.4 Steroid Hormones

Estrogen deficiency causes osteoporosis as does chronic treatment with high doses of corticosteroids (Hahn, 1989; Ericken et al., 1990). In experimental animals (Turner et al., 1987) and humans (Riggs et al, 1972; Lindsay et al., 1980) the major effect of estrogen is to decrease
bone resorption in vivo. This antiresorptive effect is followed by a delayed decrease in bone formation. However, estrogens display minimal effects on bone in vitro. Radioimmunoassays for estrogen receptors have demonstrated their presence in human osteoclasts (de Vernejoul et al., 1988) but their functional significance has not been established. Therapeutic doses of corticosteroids in vivo increase bone resorption (Hahn et al., 1974; Dykman et al., 1985) as well as serum PTH concentrations (Dykman et al., 1985). This suggests that a portion of the increased resorption seen with corticosteroid treatment may be due to the induction of higher PTH levels.

1.5 LOCAL FACTORS

Systemic and local regulators of bone resorption complement each other. The potential for complex interactions of local and systemic factors in osteoclast regulation is vast. The challenge is to determine the composition of the cellular environment, whether it is an interaction between cells, between cells and the extracellular matrix or soluble systemic and local factors that are responsible for the coordinated processes of resorption and formation of bone matrix. The local factors, interleukin-1, interleukin-6, tumour necrosis factor, prostanoids and lipoxygenases, platelet activating factor and reactive molecules, oxygen and nitrogen intermediates will be reviewed.

1.5.1 Interleukin - 1

IL-1 may have an important role in a number of disease states which are characterized by large accumulations of chronic inflammatory cells producing IL-1 together with other cytokines. Gowen and co-workers (1983) first showed that natural human purified IL-1 stimulated bone resorption. The mechanism of action is indirect via initial interactions
with osteoblasts (Lorenzo et al., 1991). IL-1 - treated isolated osteoclasts do not resorb bone unless incubated in the presence of conditioned medium from IL-1 - treated osteoblasts. The nature of this osteoblast-derived factor(s) and the mechanism by which it interacts with osteoclasts and induces their activation is not clearly defined.

Some actions of IL-1 may involve other cytokines such as IL-6. The addition of IL-6 antibodies decreases the action of IL-1. Thus it seems that IL-6 mediates some of IL-1’s actions on bone (Garrett et al., 1990). In addition IL-1 acts in conjunction with other factors to mediate its effect on bone and calcium homeostasis. Dewhirst et al. (1987) demonstrated that PTH and PTH-related protein (Sato et al., 1989) produced synergistic effects on osteoclastic bone resorption when added together with IL-1.

There are two types of IL-1; IL-1 alpha and IL-1 beta. Both of the IL-1 molecules have equivalent effects on bone resorption both in vitro and in vivo (Gowan 1983, Dewhirst et al., 1985). Recently, an IL-1 receptor antagonist (IL-RA) has been purified and found to have considerable homology with IL-1. IL-RA blocks the actions of IL-1 by blocking the receptor (Arend et al., 1989). Since IL-1 is likely to be an important mediator of osteoclastic bone resorption, IL-RA may have considerable therapeutic potential in those circumstances where IL-1 is the major mediator involved.

1.5.2 Interleukin-6

IL-6 is an unique cytokine which not only affects bone cells but is also produced by bone cells (Feyen et al., 1989). Lowik et al. (1989) showed that IL-6 increased osteoclastic activity in a murine model and Kurihara et al. (1990) showed that IL-6 induced osteoclast formation in marrow cultures. Furthermore, Mundy et al. (1992) showed that the loss of trabecular bone associated with bone resorption occurred when Chinese hamster ovary cells transfected with IL-6
were transplanted in nude mice. Again, there are complex interactions between IL-6 and other cytokines. IL-6 levels can be modulated by PTH, IL-1 and TNF (Feyen et al., 1989). Furthermore, IL-6 can potentiate the effects of IL-1 and TNF on the induction of resorptive activity (Mundy, 1992). Currently it is still unclear whether the effects of IL-6 are direct or indirect.

1.5.3 Tumour Necrosis Factors

Tumour necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) are both capable of inducing bone resorption (Bertolini et al., 1986). These factors are likely involved in bone physiology, both in normal and pathological conditions. TNF-alpha is produced by activated monocytes and macrophages while TNF-beta is produced by activated T-lymphocytes (Mundy, 1992). Pfelschifter et al. (1989) studied the effects of these cytokines in marrow cultures and found that they increased osteoclast numbers. The mechanism of action proposed was the stimulation of progenitor cells to proliferate and then promote differentiation and fusion of committed progenitors to form mature multinucleated cells. Contrariwise, this effect may be indirect as Thomson et al. (1987) showed that tumour necrosis factor induces osteoblastic cells to stimulate bone resorption. Complex interactions exist between TNF and other cytokines. The effects of TNF on bone resorption are inhibited by antibodies to IL-1 receptor and to IL-1 RA (Garrett et al., 1990, Black et al., 1990) and can partially be inhibited by antibodies to IL-6.

1.5.4 Platelet Activating Factor

Platelet activating factor (PAF) is an inflammatory mediator secreted by activated macrophages and granulocytes. PAF acts directly on isolated rat osteoclasts to elevate cytosolic free Ca++ concentration and stimulate bone resorption (Zheng et al., 1993). Evidence indicative
of direct effects has been shown by Gravel et al. (1994). They demonstrated that PAF directly induced pseudopod formation which is a morphological response characteristic of osteoclast activation.

1.5.5 Nitric Oxide

Nitric oxide (NO) is a small relatively unstable potentially toxic diatomic free radical (Feldman et al., 1993). This inorganic gas affects an astonishing range of physiological processes. Nitric oxide appears to diffuse freely in all directions from its site of origin, making control of its synthesis key to regulating its activity. Macrophages have been shown to release the cytotoxic short lived reactive radical nitric oxide in response to inflammatory cytokines (Hibbs et al., 1991). Schmidt et al. (1992) demonstrated that the enzyme responsible for nitric oxide production (nitric oxide synthase) was present in areas of bone corresponding with osteoclasts and bone remodelling activity. They believe that osteoclasts make NO and also serve as targets of NO action. MacIntyre et al. (1992) reported that NO generating agents caused a decrease in isolated rat osteoclasts cell spread area and bone resorption. In addition, inhibition of nitric oxide has been shown to inhibit the parathyroid hormone -1,25 di-hydroxyvitamin D₃- induced stimulation of resorption in a 19 day fetal rat limb resorption assay system (Stern et al., 1992).

Potentiation of osteoclastic bone resorption activity by inhibition of nitric oxide synthase (NOS) has been shown. Kasten et al. (1991) have examined the effects of modulating nitric oxide levels on osteoclast mediated bone resorption in vitro and the effects of nitric oxide synthase inhibitors on bone mineral density in vivo. Chicken osteoclasts were cultured on bovine bone slices in the presence or absence of the NO generating agent, sodium nitroprusside, or the NOS inhibitors N-nitro L-arginine methyl ester and aminoguanidine. Nitroprusside markedly decreased the number of resorption pits and the average pit area in comparison with control cultures. On
the other hand, NOS inhibition by N-nitro L-arginine methyl ester of aminoguanidine dramatically increased the number of resorption pits and the average resorption area per pit. These findings suggest that endogenous NO production in osteoclast cultures may regulate resorptive activity and that modulation of NOS and NO levels by cells within the bone microenvironment may be a sensitive mechanism for local control and prevention of osteoclastic bone resorption.

1.5.6 Prostaglandins

Prostaglandins are products of arachidonic acid metabolism which are formed by the action of cyclooxygenases. The cyclo-oxygenases initiate a cyclic pathway leading to prostaglandins, thromboxanes and prostacyclins. There are two forms of cyclo-oxygenases (COX-1 AND COX-2). Functionally, both forms are inhibited by acetylsalicylic acid. The COX enzymes catalyze the stereospecific insertion of molecular oxygen into various positions in the arachidonic acid molecule. The insertion of oxygen is the energy currency of this large family of molecules each resulting in an unique spectrum of biological activity. Prostaglandins, specifically of the E series (PGE₂) stimulated osteoclastic bone resorption in organ cultures (Klein et al., 1970; Tashjian et al., 1972) but paradoxically inhibited the activity of isolated osteoclasts in culture (Arnett et al., 1987; Fuller & Chambers, 1989; Chambers et al., 1985; de Vernejoul et al., 1988).

In isolated cell-culture systems, Chambers et al. (1983) showed that PGEs cause contraction of the cytoplasmic membrane resulting in inhibition of motility and inhibition of bone resorption. Bergmann, et al. (1995) has reviewed several other prostaglandins and their stimulation of bone resorptive activity in bone organ cultures. The ranking of the prostaglandins potencies was PGE₂>PGI₂>PGF₂alpha>PGD₂ with respect to stimulation of bone resorption in
bone organ cultures. However a PGI₂>PGE₂>PGF₂alpha>PGD₂ ranking was found for the inhibition of isolated osteoclast activity. Chambers discussed in his initial report (Chambers et al., 1983) that PGs effects may be transient and that the overall effect of prostaglandins on bone resorption may be stimulatory. This was further investigated by Okuda et al. (1989), who found that in short-term cultures, PGE₂ inhibited osteoclast activity, but then its activity was stimulatory in long-term cultures.

1.5.7 Leukotrienes

Leukotrienes are products of arachidonic acid conversion via lipoxygenase pathways. They are a family of 5-lipoxygenase (5-LO) enzyme metabolites of arachidonic acid and include the peptide (LTC₄, LTD₄, LTE₄) and non-peptide leukotrienes (LTB₄), the hydro-peroxy and hydroxyeicosatetraenoic acids (HPETEs and HETEs) and the lipoxins. Gallwitz et al., 1993 have shown that 5-LO metabolites stimulate resorption in osteoclasts isolated from osteoclastomas.

The family of lipoxygenase enzymes includes 15-LO, 12-LO and 5-LO. These enzymes oxidatively metabolize a wide range of unsaturated fatty acids. The 5-LO enzyme has been purified to homogeneity from several sources and in each case activity is dependant on Ca²⁺ and ATP, a feature that distinguishes the enzyme from the other lipoxygenases. They possess a molecular mass ranging from 74-80 kDa and an amino acid sequence that is well conserved throughout mammalian species. For example, the amino acid sequence of human and rat 5-LO enzyme derived from their cDNA clones shows 93% identity. (Dixon et al., 1988, Balcaret et al 1988). Lipoxygenases and other enzymes that metabolize arachidonic acid are selectively distributed in different cell types, further increasing the diversity of the eicosanoids that can be produced.

Arachidonic acid, when released from cell membranes by the action of phospholipase A₂,
is converted by the enzyme 5-lipoxygenase to 5S-hydroperoxy, 6,8-trans 11,14 cis eicosatetraenoic acid (5-HPETE). The same enzyme catalyzes the conversion of 5-HPETE to leukotriene A₄. LTA₄ can then be converted to leukotriene B₄ or conjugated with reduced glutathione to form leukotriene C₄. LTC₄ and its products, leukotriene D₄ and leukotriene E₄ make up the biologic mixture previously known as the slow reacting substance of anaphylaxis. 5-LO is a non-heme iron containing enzyme. In human leukocytes, 5-lipoxygenase catalyzes the oxygenation of arachidonic acid to 5-HPETE and the dehydration of 5-HPETE to LTA₄ as it's first committed step in the synthesis of leukotrienes (see Yamamoto et al. 1992 for review). 5-LO in human leukocytes is a soluble 78-kDa protein which becomes membrane bound and activated following cell activation by various stimuli that raise intracellular calcium levels. The activity of purified enzyme is strongly stimulated by physiological concentrations of calcium and to a lower extent by ATP and also by phospholipid vesicles or detergent micelles which presumably mimic the membrane environment in which cellular arachidonic acid oxidation occurs. (Deniss et al. 1991; Percival et al., 1992). All lipoxygenases characterized so far, including 5-lipoxygenase (Percival, 91) have been shown to contain an essential iron atom which is believed to cycle between the ferric and ferrous forms during the catalytic cycle.

Five Lipoxygenase activating protein (FLAP) is an 18-kDa membrane bound protein required for leukotriene biosynthesis in intact cells, (Ford-Hutchinson 1991). In light of their key role in leukotriene biosynthesis both 5-LO and FLAP have been pharmacological targets for drug development.

Unlike 5-lipoxygenase, which is selectively distributed, LTA₄ hydrolase, the enzyme that converts LTA₄ to LTB₄, is widely distributed and is ubiquitous in mammalian tissues (Samuelson et al., 1987). For many years LTA₄ hydrolase was believed to reside exclusively in white blood cells, especially granulocytes. When enzymatic activity was found in mammalian plasma and
subsequently in almost all tissues and cells examined, this notion was revised. Even some cells which do not express 5-LO activity and therefore are unable to synthesize the substrate LTA₄ harbour LTA₄ hydrolase activity. Recently, cooperation between cells on a the processing of reactive precursors and intermediates of arachidonic acid metabolism into biologically active compounds has emerged as an important concept. Several studies in vitro have shown that LTA₄ can be formed in a donor cell and then transferred to a recipient cell for further conversions (Dahinden et al. 1989). Thereby, surrounding cells, devoid of 5-Lipoxygenase activity but expressing LTA₄ hydrolase and or LTC₄ synthase, could nevertheless participate in leukotriene production. Since several cells have only LTA₄ hydrolase and lack 5-LO (Fitzpatrick et al. 1984, Claesson et al. 1988) therefore, the actual activity exhibited in a given system, may be governed by the availability of the substrate LTA₄.

LTA₄ has a very short half life in aqueous solution (<10 seconds) and is extremely labile in aqueous media. The major hydrolysis products of LTA₄ are 5,12 diHETEs which are produced in equal amounts during arachidonic acid oxidation by leukocytes and in vitro by purified 5-LO. LTA₄ is also hydrolysed to yield smaller amounts of 5,6 diHETEs. These nonenzymatic hydrolysis products have little biological activity as compared to LTB₄ and the peptidoleukotrienes and no function has been assigned to them. However their presence in biological material reflects 5-lipoxygenase activity.

1.6 OBJECTIVES

Previous studies by Gallwitz et al. (1993) have shown that metabolites of the 5-lipoxygenase pathway stimulated formation of osteoclasts and may regulate osteoclast function. Meghji et al.(1988) reported that lipoxygenase metabolites stimulated bone resorption in calvaria cultures. These two experiments suggested that products of the 5-LO pathway may be major
mediators of stromal cell-induced osteoclastic activity. Other reports indicated that products of the 5-LO pathway, particularly LTB₄ were associated with a number of disease processes characterized by significant bone loss (Et Attar et al., 1983, Porteder et al., 1984; Keppler, 1992). Since LTB₄ production is elevated in inflammatory conditions, it is likely involved in the bone loss observed when chronic inflammatory tissues lie adjacent to bone surfaces. It is believed that blocking LTB₄ activity may have therapeutic utility for treatment of a variety of inflammatory conditions (McMillan & Foster, 1988). Other leukotrienes that could be possible mediators were the peptidoleukotrienes (LTC₄, LTD₄ and LTE₄). However, based on the findings that LTB₄ concentrations were significantly higher than peptidoleukotrienes at sites of inflammation (Rae et al., 1982; Davidson, et al., 1983), we selected LTB₄ for our investigations.

Our objective was to evaluate the role of LTB₄ with respect to formation of osteoclasts and osteoclast precursors and the resorptive activity of differentiated osteoclasts with the idea in mind that LTB₄ might be a good candidate for being an osteoblast-produced direct activator of osteoclastic activity. To distinguish between direct and indirect effects of LTB₄ on osteoclastic activity and/or formation, we used isolated rabbit osteoclasts co-cultured with or without added osteoblast-like cells (UMR 106-01). Lastly, we examined the effect of blocking LTB₄ mediated effects through the use of a specific LTB₄ receptor antagonist LY 292 728 (Sawyer et al., 1995).
CHAPTER 2

THE EFFECT OF SERUM CONCENTRATION IN THE CULTURE MEDIUM ON THE NUMBER AND SIZE OF OSTEOCLASTS
2.1 INTRODUCTION

To be able to test the effects of LTB₄ on the number and the activity of osteoclasts, culture conditions that allow us to study its action have to be established. As discussed in the general introduction, LTB₄ is extremely labile. The half-life of LTB₄ in vivo is 38 seconds in rat and 4 minutes in human (Keppler et al., 1992). In addition, serum in culture medium accelerates the degradation of LTB₄ and binding to albumin inactivates it (Keppler et al., 1992). Others who have investigated effects of leukotrienes have emphasized that serum concentrations should be lower than 5% to minimize its inactivation (Gallwitz et al., 1993). Since standard osteoclast culture procedures usually employ 10% fetal calf serum in culture media (Chambers et al., 1984), we compared the number and size of TRAP + multinucleated cells, the number of mononuclear TRAP+ cells and stromal cell numbers in osteoclast-containing cultures maintained for 48 hours in culture media containing 1%, 2% and 10% serum to determine the minimum serum concentration in the medium that would still allow formation of multinucleated TRAP+ cells and measurement of their resorptive activity.
2.2 MATERIALS AND METHODS

2.2.1 Rabbit Osteoclast Preparation

Osteoclasts were isolated from one day old New Zealand White rabbits as described by Chambers et al., (1984). The long bones were dissected free of attached soft tissues and placed into medium 199 (Gibco BRL, Life Technologies, Grand Island NY) and supplemented with 10% heat inactivated fetal calf serum (FCS) (Bio Whittaker Walkersville, Maryland) and antibiotics (100ug/ml penicillin G, Sigma Chemicals, Inc., St. Louis, MO.) (50 ug/ml gentamycin and 0.3 ug/ml fungizone - Gibco BRL, Life Technologies, Grand Island NY) The long bones were cut longitudinally and mechanically curetted in 10 ml medium 199 + 10% FCS + gentamycin, penicillin G and fungizone. The fragments were then vigorously agitated by pipetting up and down using a pasteur pipette. The fragments were allowed to settle and the cell suspension was then transferred to a 50ml tube and the previous step repeated three times to a total volume of 40ml. This was then pipetted up and down two or three times, larger fragments were allowed to settle and the cell suspension transferred to a fresh 50 ml tube and centrifuged at 200G for eight minutes. The supernatant was removed and the pellet was immediately resuspended in alpha-Minimal Essential Medium (alpha-MEM + RNA-DNA, Flow Laboratories, Inc., McLean, VA) with 10% FCS and antibiotics. A 50uL droplet of the final cell suspension was plated onto 35 mm Falcon tissue culture plastic dishes (Falcon labware, Becton Dickinson, Lincoln Park, NJ.) and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and at pH 7.4. Ninety minutes was allowed for cell attachment, after which 2ml of culture medium was added and culture continued.
2.2.2 Culture Conditions

To test the effect of serum concentration, osteoclasts were plated in bicarbonate buffered medium with 10% serum on tissue culture plastic for 1.5 h, after which medium containing 10% serum was added. After a further 18 hours of culture, unattached cells were removed by washing. Washing consists of gently rinsing the cells in two changes of medium. The medium was replaced with medium supplemented with 1%, 2% or 10% serum and culture continued for an additional 48 hours. To test the effect of time of removal of unattached cells, osteoclasts were plated and washed after 1.5 hours or after 19.5 hours. Regardless of time of washing medium was replaced with medium supplemented with 1%, 2% or 10% serum after 19.5 hours and culture continued for an additional 48 hours. We also performed a series of experiments in which osteoclasts were plated in bicarbonate buffered medium with 2% serum on tissue culture plastic for 1.5 h at which time medium containing 2% or 10% serum was added. After 18 hours, non-adherent cells and red blood cells were washed in two changes of medium, after which medium containing 2% or 10% serum was again added and culture continued for an additional 48 hours. When referring to percentage (%) of serum in medium at plating, overnight attachment and 48 hour culture, the groups are referred to as 2,2,2; 2,10,2; 2,10,10. All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and at pH 7.4.

After culture, adherent cells were fixed with 10% neutral buffered formalin for fifteen minutes at room temperature and washed twice in distilled water. They were then stained for tartrate resistant acid phosphatase (TRAP) activity by incubating with AS-BI phosphate as substrate in Michaelis-Veronal Acetate buffer at pH 5.0 in the presence of 50 mM L-Tartaric acid. Hexazonium pararosanilin was used as a coupling agent (Barka & Anderson, 1962). After repeated washings with distilled water, the cultures were counter-stained with dilute haematoxylin to stain stromal cells. TRAP + cells were stained red and stromal cells were purplish/grey.
2.3.4 **Evaluation of Cultures**

TRAP positive mononuclear and multinucleated cells were counted using light microscopy (Leitz Makroskop M420, Germany) at a magnification of 35X. Cells containing two or more nuclei that were stained with TRAP were classified as TRAP positive multinucleated cells (TRAP + MNC). Morphology assessments were based on qualitative assessments of osteoclast size, nuclear content and shape. Numbers of stromal cells were also based on qualitative assessments of numbers of stromal cells per microscopic field.

2.3.4 **Statistical Analysis**

Statistical analysis was performed for each experiment using the two tailed unpaired Student's t-test.
2.3 RESULTS

2.3.1 Effect of the serum concentration in the culture medium on formation, survival and morphology of TRAP + cells.

Both the numbers of TRAP+multinucleated cells and TRAP+mononuclear cells were affected by the serum concentration of the culture medium. Cell numbers were highest with 10% serum and decreased with decreasing serum concentration (Figure 2.2).

Figure 2.3 shows representative pictures of osteoclasts cultured for the last 48 hours of culture in medium containing three different concentrations of serum. In 10% serum, most of the osteoclasts were large and well-spread. They contained greater than 10 nuclei contain and were surrounded by a dense layer of stromal cells (Figure 2.3.A). In 2% serum the number of large osteoclasts decreased and most osteoclasts were somewhat smaller, containing less than 10 nuclei per cell. The number of stromal cells per microscopic field were reduced compared to cultures in 10% serum (Figure 2.3.B). When cultured, in 1% serum, the number of large osteoclasts and stromal cells was further decreased compared to those cultured in 2% serum (Figure 2.3.C).
Schematic representation of culture conditions for the various serum experiments. Osteoclast containing populations were plated onto plastic tissue culture dishes in bicarbonate buffered medium. The pH of the culture media was 7.4.

A. Effect of serum concentration: Osteoclasts were plated in bicarbonate buffered medium with 10% serum on tissue culture plastic. After 1.5 hours, 2ml medium containing 10% serum was added and culture continued for 18 hours. After 18 hours of culture cells were washed to remove non-adherent cells and the medium was replaced with medium supplemented with 1%, 2%, or 10% serum. Culture was then continued for an additional 48 hours.
CULTURE CONDITIONS

A: EFFECT OF SERUM CONCENTRATION

50μl of osteoclast-containing suspension plated in bicarbonate buffered medium with 10% FCS

1.5 hour

2ml of 10% FCS was added

18 hours

Cells were washed, then medium was replaced with 1%, 2% or 10% serum.

1% serum 2% serum 10% serum

All cultures continued for 48 hours
Fig 2.1.B  *Culture Conditions*

Schematic representation of culture conditions for the various serum experiments. Osteoclast containing populations were plated onto plastic tissue culture dishes in bicarbonate buffered medium. The pH of the culture media was 7.4.

B. Effect of time of washing away non adherent cells: Osteoclasts were plated in bicarbonate buffered medium with 10% serum on tissue culture plastic. After 1.5 hours, 2ml of medium containing 10% serum was then added and culture continued for 18 hours. Non-adherent cells were washed away after 1.5 hours of after 19.5 hours. Medium was replaced with medium supplemented with 1%, 2% or 10% serum after 19.5 hours and culture continued for an additional 48 hours.
CULTURE CONDITIONS

B: EFFECT OF REMOVAL OF UNATTACHED CELLS AT DIFFERENT TIMES

50\mu l of osteoclast-containing suspension plated in bicarbonate buffered medium with 10% FCS

1.5 hour

2ml of 10% FCS was added

1.5 hour

Cells were washed, then 2ml of 10% FCS was added

2ml of 10% FCS was added

18 hours

Cells were washed, then 2ml of 1% FCS was added

18 hours

medium replaced with 1%, 2%, 10% FCS

1% serum 2% serum 10% serum 1% serum 2% serum 10% serum

All cultures continued for 48 hours
Fig 2.1.C  *Culture Conditions*

Schematic representation of culture conditions for the various serum experiments. Osteoclast containing populations were plated onto plastic tissue culture dishes in bicarbonate buffered medium. The pH of the culture media was 7.4.

C. Effect of plating the cells in medium containing 2% serum: Osteoclasts were plated in bicarbonate buffered medium with 2% serum on tissue culture plastic for 1.5 h after which 2mls of 2% or 10% supplemented medium was added and culture continued for 18 hours. At 19.5 hours of culture, non adherent cells were washed away. The medium was then replaced with medium supplemented with 2% or 10% serum. Cultures were continued for an additional 48 hours. The conditions for the different cultures are indicated in the figure as 2,2,2, 2,10,2: 2,10,10. (Serum concentration of medium in the first 1.5 hours, the next 18 hours, and following 48 hours respectively)
C: THE EFFECT OF PLATING THE CELLS IN MEDIUM CONTAINING 2% SERUM

50μl of osteoclast-containing suspension plated in bicarbonate buffered medium with 2% FCS

1.5 hour

1.5 hour

2ml of 2% FCS was added

2ml of 10% FCS was added

18 hours

18 hours

Cells were washed, then 2ml of 2% FCS was added

Cells were washed, then 2ml of 2% or 10% FCS was added

2% serum
(2, 2, 2)

2% serum
(2, 10, 2)

10% serum
(2, 10, 10)

All cultures continued for 48 hours
The Effect of Serum Concentration on Numbers of Osteoclasts

Osteoclast containing populations were plated on plastic tissue culture dishes in bicarbonate buffered medium with 10% serum. After 1.5 hours, 2ml medium containing 10% serum was added and cultures continued for 18 hours. After 18 hours of culture cells were washed to remove non-adherent cells and the medium was replaced with medium supplemented with 1%, 2% or 10% serum. Culture was then continued for an additional 48 hours. The pH of culture media was 7.4. Results are expressed as mean number of TRAP+MNC or TRAP+mononuclear cells per culture dish ± sem for fourteen dishes.

* = significantly different from 10% serum, p<0.05 using Student’s t-test
# = significantly different from 2% serum, p<0.05 using Student’s t-test
TRAP + mono

TRAP + MNC

1 2
(Oh)
FCS
In
Culture
10
Medium

Numbers of TRAP+ Cells

(% FCS In Culture Medium)
Osteoclast containing populations were plated on plastic tissue culture dishes in bicarbonate buffered medium with 10% serum. After 1.5 hours, 2mls of medium containing 10% serum was added and cultures continued for 18 hours. After 18 hours of culture cells were washed to remove non-adherent cells and the medium was replaced with medium supplemented with

A: 10% serum
B: 2% serum
C: 1% serum

Culture was then continued for an additional 48 hours. Microscopical pictures at 35X magnification.
2.3.2 Effect of Time of Removal of Unattached Cells

Removal of unattached cells after 1.5 hours significantly reduced the numbers of TRAP-positive multinucleated and mononuclear cells in cultures containing medium with 2% serum or 10% serum (figure 2.4).

Representative pictures are shown in Figure 2.5. Removal of unattached cells after 1.5 hours appeared to reduce osteoclast size and also reduced the number of stromal cells per microscopical field compared to cultures where unattached cells were removed at 19.5 hours.
The Effect of Removal of Unattached cells at different Times on Numbers of Osteoclasts

Osteoclast containing cell populations were plated in bicarbonate buffered medium with 10% serum on tissue culture plastic. After 1.5 hours, 2ml medium containing 10% serum was added and culture continued for 18 hours. Non adherent cells were washed away after 1.5 hours or after 19.5 hours. Medium was replaced with medium supplemented with 1%, 2% or 10% serum after 19.5 hours and culture continued for an additional 48 hours. Results are expressed as mean number of TRAP+MNC and TRAP+mononuclear cells per tissue culture dish ± sem for six dishes.

* = significantly different from 1% - 19.5 hour washing, p<0.05 using Student’s t-test
+ = significantly different from 2% - 19.5 hour washing, p<0.05 using Student’s t-test
# = significantly different from 10% - 19.5 hour washing, p<0.05 using Student’s t-test
Fig 2.5  *The Effect of Removal of Unattached cells at different times on Numbers and Morphology of cells*

Osteoclast containing cell populations were plated in bicarbonate buffered medium with 10% serum on tissue culture plastic. After 1.5 hours, 2ml medium containing 10% serum was then added and culture continued for 18 hours. Non adherent cells were washed away after 1.5 hours or after 19.5 hours. Medium was replaced with medium supplemented with 1%, 2% or 10% serum after 19.5 hours and culture continued for an additional 48 hours. Microscopical pictures at 35X magnification.

A: 10% serum 19.5 h wash  
C: 2% serum, 19.5 h wash  
E: 1% serum, 19.5 h wash  
B: 10% serum, 1.5 h wash  
D: 2% serum, 1.5 h wash  
F: 1% serum, 1.5 h wash
2.3.3 Effect of plating the cells in medium containing 2% serum.

Cultures were plated in 2% serum. After 1.5 hours, medium was replaced with 2% or 10% serum and continued until 19.5 hours, after which time medium was replaced again with medium containing 2% or 10% serum. The numbers of TRAP+mononuclear and TRAP+multinucleated cells at the end of the culture period under the different conditions is shown in Figure 2.6. The rank order of TRAP+mononuclear cells was 2,10,10 > > 2,10,2 > 2,2,2. With respect to multinucleated cells, the order was 2,10,10 > 2,10,2 = 2,2,2. Microscopical examination of morphology in the three different culture conditions indicate that the presence of 10% serum during any time period in culture resulted in abundant stromal cells (Figures 2.7). Use of 2% serum throughout the duration of culture period resulted in fewer stromal cells than in cultures with 10% serum.
The Effect of Plating the cells in medium containing 2% serum.

Osteoclast containing cell populations were plated in bicarbonate buffered medium with 2% serum on tissue culture plastic for 1.5 hours after which 2mls of 2% or 10% supplemented medium was added and culture continued for 18 hours. At 19.5 hours of culture, non adherent cells were washed away. The medium was then replaced with medium supplemented with 2% or 10% serum. Cultures were continued for an additional 48 hours. The conditions for the different cultures are indicated in the figure as 2,2,2: 2,10,2: 2,10,10. (serum concentration of medium in the first 1.5 hours, the next 18 hours, and following 48 hours respectively.) Results are expressed as mean number of TRAP+MNC and TRAP+mono cells per dish ± sem for six dishes

* = significantly different from 2,10,2, p<0.05 using Student’s t-test
+ = significantly different from 2,10,10, p<0.05 using Student’s t-test
TRAP +ve
TRAP +ve
MNC

2,2,2
2,10,2
2,10,10

FCS In Culture Medium

Numbers of TRAP+ Cells

(%) FCS In Culture Medium

TRAP +ve mono
TRAP +ve MNC

* +

* +

* +

* +

* +

* +
The Effect of Plating the cells in medium containing 2% serum on Morphology of cells

Osteoclast containing cell populations were plated in bicarbonate buffered medium with 2% serum on tissue culture plastic for 1.5 hours after which 2mls of 2% or 10% supplemented medium was added and culture continued for 18 hours. At 19.5 hours of culture, non adherent cells were washed away. The medium was then replaced with medium supplemented with 2% or 10% serum. Cultures were continued for an additional 48 hours. The conditions for the different cultures are indicated in the figure as serum concentration of medium in the first 1.5 hour, the next 18 hours and the following 48 hours respectively. Microscopical pictures at 35X magnification.

A) 2, 10, 10
B) 2, 10, 2
C) 2, 2, 2
DISCUSSION

The purpose of these experiments was to establish satisfactory low serum conditions to allow us to study the effects of LTB₄ on osteoclast activity and osteoclast formation. In the first series of experiments we compared the effect of serum concentration on numbers of osteoclasts. We found that higher serum concentration increased osteoclast numbers which was particularly evident when cultures with 10% serum were compared to those in 1% serum.

With respect to morphology, the size of the osteoclasts formed also increased with increasing serum concentration. Cultures with 10% serum contained large multinucleated cells with light staining for TRAP. In contrast, cultures with 1% or 2% serum generally contained smaller, darkly stained multinucleated osteoclasts. The cells cultured in 2% serum were larger than those in 1% serum but smaller than those in 10% serum. The use of 2% serum resulted in the formation of both mononuclear and of multinucleated osteoclasts that ranged in size from small to large. Thus, culture media containing 2% serum seem to be optimal to study effects of agonists on osteoclasts formation.

Evaluation of stromal cell growth revealed a strong dependence on serum. Stromal cells were abundant in 10% cultures and reduced in 1% and 2% cultures. In the second series of experiments, we found that removal of unattached cells after 1.5 hours significantly reduced the numbers of TRAP+ cells and stromal cells when compared to removal of unattached cells after 19.5 hours of culture. A likely explanation for this decrease is that after 1.5 hours some mononuclear TRAP+ cells are less firmly attached than at 19.5 hours of culture. This explanation would be consistent with our observations that reduced number, size and multinuclearity of TRAP+MNC cells are found in cultures with the removal of unattached cells at 1.5 hours.

Lastly, the evaluation of cultures in which cells were plated in medium containing 2%
serum showed that using 10% serum during the 18 hour time period resulted in increased osteoclast numbers when compared to those cultured with 2% serum. However, cultures treated with 10% serum contained many stromal cells. Cultures plated in medium containing 2% serum and cultured under these conditions throughout the experiment (i.e. 2,2,2) resulted in good osteoclast number/survival and relatively low stromal cell numbers.

In summary, based on numbers of osteoclasts, stromal cells, mononuclear TRAP+cells and on the morphology of osteoclasts, plating and culture in medium containing 2% serum, and removal of unattached cells after 19.5 hours were selected as standard culture conditions for future experiments.
CHAPTER 3

THE EFFECT OF LTB₄ ON DEVELOPMENT OF TRAP⁺ CELLS, NUMBERS OF RESORPTION LACUNAE AND TOTAL AREA OF BONE RESORBED IN OSTEOCLAST CULTURES AND IN CO-CULTURES OF OSTEOCLASTS AND UMR 106-01 CELLS
3.1 INTRODUCTION

As reviewed in the introduction of this thesis, LTB₄ is a powerful but short-lived derivative of oxidative arachidonate metabolism. LTB₄ is chemotactic for polymorphonuclear leucocytes, eosinophils, lymphocytes and monocytes and increases adherence, oxygen radical production and lysosomal degranulation in polymorphonuclear leucocytes (Goldman et al., 1986). A number of cytokines and growth factors such as interleukin-1 and interleukin-2 induce the production and secretion of leukotrienes (Parker, 1987).

Gallwitz et al. (1993) discovered that products of the 5-LO pathway directly stimulated osteoclastic resorption by dispersed rat and human osteoclasts cultured on bone slices. These results and the report by Meghji et al. (1988) that LO metabolites stimulated bone resorption in calvarial cultures provided strong evidence that LO pathway metabolites could have a role in the physiological regulation of osteoclastic activity. There are also reports that lipoxygenase metabolites of arachidonic acid (AA) are involved in bone loss related to periodontal diseases (El Attar and Lin, 1983) and oral cancers (Porteder et al., 1984). In inflamed periodontal tissues lipoxygenase activity exceeds cyclooxygenase activity (Sidhagea et al., 1982), and leukotrienes appear to be the major products of AA metabolism (Klickston et al., 1980, Matejka et al., 1985). There is evidence that leukotrienes are formed by human marrow cells, and that increased production of LTB₄ in chronic myelocytic leukemia and in inflammatory diseases is associated with bone resorption (Stenke et al., 1987, Keppler, 1992). Furthermore, there are reports that leukotrienes are produced by bone (Collins et al., 1987, Offenbacher et al., 1986) and osteoblastic cells (Zhang & Dziak, 1990).

Bone resorption is regulated by the generation of new osteoclasts and the activation of existing osteoclasts. As discussed in the general introduction, many bone resorbing cytokines and hormones do not act directly on osteoclasts but rather on their precursors in the bone marrow
environment or on osteoblasts.

In this chapter we present experiments evaluating the effect of leukotriene B4 on the formation of TRAP + cells and resorption lacunae using isolated osteoclasts cultured on bone slices at pH 7.4. Since the resorptive activity of osteoclasts is increased at pH 7.0 (Arnett & Dempster, 1986; Shibutani & Heersche, 1993) we also investigate the possibility that the effects of LTB₄ may be more pronounced at pH 7.0. Furthermore, we evaluate the possibility that leukotrienes act indirectly on osteoclasts via effects on osteoblast-like cells and stromal cells, using co-cultures of osteoblast-like cells (UMR 106-01) and osteoclasts.
3.2 MATERIALS AND METHODS

3.2.1 Rabbit Osteoclast Isolation and Culture Conditions

Rabbit osteoclasts were isolated as described in Chapter two. Based on the results presented in chapter 2, the culture conditions chosen were the following:

Neonatal rabbit osteoclasts were prepared and plated on either 35 mm tissue culture dishes or on 6mm diameter devitalized cortical bone slices in 96 well microtiter plates in bicarbonate buffered α-MEM with 2% FCS and antibiotics (standard medium).

35 mm Tissue Culture Dish: The cells plated in culture dishes were allowed ninety minutes for attachment before the addition of 2 mls of standard medium. After 18 hours of culture, cells were washed to remove non-adherent cells. The medium was then replaced with control or LTB$_4$ supplemented medium and cultures continued for an additional 48 hours.

Devitalized Cortical Bone Slices: Bone slices were placed in the wells of 96 well microtiter plates and 150uL of standard medium added. Then 50 uL of cell suspension was added. After 18 hours of culture, medium was removed and replaced with fresh medium with or without LTB$_4$ and cultures continued for 24 h or 48 h. All cells were cultured at 37°C in a humidified atmosphere of 95 % air and 5 % CO$_2$ in standard medium at pH 7.4 (unless otherwise stated).

In some cultures, osteoclasts were maintained in medium at pH 7.0. For these cultures, α-MEM without bicarbonate (Flow Laboratories Inc., McLean, VA) containing 25 mM HEPES (Sigma Chemicals Inc., St. Louis, Mo.)+2% FCS and antibiotics was used. The pH was adjusted to pH 7.0 with 0.1 N HCL as required. Before adding cells, freshly prepared medium was incubated for 20 h under culture conditions (humidified air, 37°C) to attain the required pH (7.0).
3.2.2 Alkaline Phosphatase and TRAP staining

After culture, cells plated on dishes were fixed with 10% neutral buffered formalin for 30 minutes at 4°C, washed twice in distilled water and stained for alkaline phosphatase activity by incubating for 30 minutes in Tris buffer (0.2M, pH 8.3) with AS-MX phosphate as a substrate and Fast Blue B salt as a stain (Burstone, 1960). After repeated washing with distilled water, the cultures were also stained for TRAP activity by incubating with AS-BI phosphate as substrate in Michaelis Veronal Acetate buffer at pH 5.0 in the presence of 50mM L-tartaric acid and with hexazonium pararosanilin as a coupling agent (30 min, 22°C). TRAP staining is a characteristic marker of osteoclast activity. Alkaline phosphatase is a characteristic cytochemical marker for osteoblasts which stain cells blue.

3.3.3 Evaluation of Cultures

At the end of the culture period the cultures were fixed and stained for tartrate resistant acid phosphatase activity (TRAP). Cells with less than 2 nuclei that stained with TRAP were classified as TRAP positive mononuclear cells (TRAP+ mono). Cells with two or more nuclei that stained with TRAP were classified as TRAP positive multinucleated cells (TRAP+MNC). In cultures where osteoclasts were plated on bone, only TRAP+MNC were assessed.

3.2.4 Staining of Resorption Lacunae

After counting the TRAP+ cells, bone slices were sonicated in PBS, and Q-tips used to scrape off osteoclasts in 0.25M ammonium hydroxide solution to remove the cells. The bone slices were then fixed in 10% formaldehyde for 6 h at 4°C and the resorption pits stained with anti-collagen type I polyclonal antibody. An antibody for type I collagen is used as a marker for bone resorption because resorbing osteoclasts release collagen from bone. Briefly, bone
slices were incubated in 3% normal sheep serum (Sigma Chemicals Inc., St. Louis, Mo.) for 30 minutes and then incubated for 2 h with primary antibody (sheep anticollagen type I antibody, gift from Dr. J. Sodek) in a 1:10 dilution in phosphate buffered saline with 1% bovine serum albumin (BSA). After washing with 0.05% Tween 20 in Tris-buffered saline, pH 7.4, the second antibody (biotinylated antisheep IgG 1:40 (Sigma Chemicals, Inc., St. Louis, Mo.) was added and the bone slices incubated for another 30 minutes. After washing, avidin-biotin complex solution (Vektastain ABC kit, Vector Lab) was added for 30 minutes. The slices were then immersed in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.03% H₂O₂ overnight. Using this technique resorption pits are seen as dark brown areas.

3.2.5 Quantitation of Bone Resorption Area and Lacunae Numbers

The number of resorption pits and the total area of resorption was quantitated manually using a Zeiss Photomicroscope II and a Zeiss Zidas system. The resorption pit area was determined by visualizing the lacunae through the Zeiss Photomicroscope and with a hand held tracing device, tracing the outline of the lacunae onto a platform which translates the tracing into an area measurement.

3.2.6 UMR 106-01 - Osteoclast Co-culture Experiments

In some experiments, osteoblastic cells were co-cultured with osteoclasts to investigate the possible indirect effects of LTB₄ on osteoclasts. All osteoblast cultures were second subcultures from frozen stock. The clonal osteoblastic osteosarcoma cell line UMR 106-01 was cultured in D-MEM (Gibco BRL, Life Technologies, Grand Island NY) + 15% FCS + AB for 48 hours after which the cells were subcultured into alpha-MEM + 10% serum +AB, and grown until 80% confluent. The cells were then trypsinized and plated on either plastic culture dishes
or onto cortical bone slices. For co-culture experiments on bone slices, bone slices were placed in the wells of 96 well microtiter plates and 150ul of medium containing UMR 106-01 cells was plated at $3 \times 10^4$ cells/cm$^2$ density and allowed to attach for 24 hours. Osteoclasts (50ul) were then settled onto the 150ul of alpha +2 %FCS + AB. After 24 hours, the medium was replaced with medium with or without LTB$_4$ and cultures continued for a further 24 hours. The cells were then fixed and stained for TRAP and resorption lacunae.

3.2.7 Pretreatment of UMR 106-01 Cells with LTB$_4$ in Osteoblast-Osteoclast Co-culture experiments

Bone slices were placed in the wells of 96 well microtiter plates and 150ul of medium with alpha-MEM + 2% FCS + AB and UMR 106-01 cells were plated onto bone slices at a density of $3 \times 10^4$ cells/cm$^2$. After 5 h of UMR cell attachment, the medium was replaced with medium with or without LTB$_4$. After 18 hours, the medium was aspirated, bone slices were rinsed briefly in two changes of fresh medium, and osteoclasts plated onto the same bone slices. The co-cultures were cultured for an additional 48 hours in the absence of LTB$_4$. The cells were then fixed, stained for TRAP and resorption lacunae.

3.2.8 The Effect of LTB$_4$ on UMR 106-01 cells

UMR cells were plated on 35 mm Falcon tissue culture dishes at a density of $3 \times 10^4$ cells/cm$^2$. After 24 hours, the medium was replaced with LTB$_4$ supplemented medium or control medium and cells were cultured for an additional 48 hours. Cell numbers were determined on days one to four of culture by trypsinizing the cells and counting the cell suspension using a Coulter-Counter (Coulter Electronics, Hiale Florida, USA).
3.2.9 **Statistical Analysis**

Most of the experiments shown were repeated at least 3 times. Some however were duplicate experiments that included 10 bone slices per treatment group per experiment. Statistical analysis was performed for each experiment using the two tailed unpaired Student's t-test.
3.3 RESULTS

3.3.1 Effects of LTB₄ on Osteoclast Numbers and Osteoclastic Bone Resorption in Osteoclast Cultures on Plastic Substrata and/or Bone.

We first tested the effects of LTB₄ on osteoclast numbers in rabbit osteoclast containing cultures on plastic substratum. The results in Fig.3.1 show that treatment with 0.1 - 1000nM LTB₄ for 48 hours produced no clear dose-dependent effect on the numbers of TRAP-positive mononuclear or multinuclear cells, although the number of TRAP+ multinucleated cells tended to be somewhat higher with the higher concentrations of LTB₄ used, while the number of TRAP+ mononuclear cells appeared to be decreased. When osteoclasts were plated on bone slices under similar culture conditions we also observed a tendency for the numbers of both TRAP+mononuclear and multinucleated cells to be increased at the higher concentrations used (Figure 3.2.A). There was no effect of LTB₄ in terms of the area of bone resorbed (Fig. 3.2.B). Based on these results, and in view of the considerable variability in the data, we re-evaluated our experimental approach and decided to focus on the effects of the higher doses of LTB₄ (100nM and 1000nM) and increase the number of data points per experiment.

The result of this next series of experiments is shown in Figure 3.3. There was a significant increase in number of osteoclasts and in the area of bone resorbed per slice with both 100nM and 1000nM (Figure 3.3.A & Figure 3.3.C). A tendency towards increased number of pits was observed (Figure 3.3.B). Recalculating these data we obtained area resorbed per osteoclast and mean pit area, but found no significant differences for these parameters (Figure 3.4.A & Figure 3.4.B). Representative photographs of osteoclasts and resorption lacunae in control cultures and cultures containing 1000nM LTB₄ are shown in Figure 3.5 and Figure 3.6. Visual observation suggests that osteoclast size and the size of the resorption lacunae area increased in cultures treated with 1000nM LTB₄. Control bone slices contained predominantly
small pits (Figure 3.6.A) while LTB₄ treated bone contained composite or tracking type pits in addition to small pits (Figure 3.6.B). Figure 3.7 shows a photograph of a stained culture where the cells were not removed. It demonstrates the high variability with respect to area resorbed by different osteoclasts: small cells resorb small areas, while large cells resorb larger areas.

Since the resorptive activity of osteoclasts is increased at pH 7.0 (Shibutani & Heersche, 1993) we considered the possibility that the effects might be more pronounced at pH 7.0. We therefore performed experiments similar to those shown in Figure 3.2 & Figure 3.3 but conducted at pH of 7.0 in HEPES buffered medium. The results were essentially similar but the differences were more pronounced. A 48 hour treatment with LTB₄ (100nM and 1000nM) significantly increased the numbers of TRAP-positive multinucleated cells, the number of pits and the total area of bone resorbed (Figure 3.8). Mean pit area was increased with 1000nM (figure 3.9). Thus lowering the pH from pH 7.4 to 7.0 increased the magnitude of the effect of LTB₄ on the area resorbed when compared to pH 7.4. Similar to the experiments conducted at pH 7.4 (Figure 3.2 & Figure 3.3), no significant effect of LTB₄ on area resorbed per osteoclast was seen at pH 7.0 (Figure 3.9)
Figure 3.1 *The Effect of LTB₄ on Numbers of Osteoclasts*

Osteoclast-containing populations were plated on plastic tissue culture dishes and cultured for 19 hours in bicarbonate buffered control medium, after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.4. Results are expressed as mean number of TRAP+ MNC and TRAP+ mono per dish +/- sem of the mean of 10 dishes:
Concentration of LTB4 (nM)

Numbers of TRAP+ Cells

- TRAP + mono
- TRAP+ MNC
Figure 3.2  
*The Effect of LTB₄ on Numbers of Osteoclasts and on Bone Resorption*

Osteoclast-containing cell populations were plated on bone slices and cultured for 19 hours in bicarbonate buffered control medium, after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.4. Results are expressed as mean number of TRAP+ mono and TRAP+MNC cells per bone slice +/- the standard errors of the mean of 8 bone slices:

A: Top Panel: **The Effect of LTB₄ on Osteoclast Numbers**

B: Bottom Panel: **The Effect of LTB₄ on Osteoclastic Bone Resorption**

* = significantly different from controls (p<0.05), using Student's t-test
TOTAL AREA OF BONE RESORBED

\( \text{\( \mu \text{m}^2 \)} \)

CONCENTRATION OF LTB4 (nM)

CONCENTRATION OF LTB (nM)

NUMBERS OF TRAP+ CELLS

TRAP+ mono

TRAP+MNC
Figure 3.3  The Effect of LTB₄ on Osteoclast Numbers and Osteoclastic Bone Resorption

Osteoclast containing cell populations were plated on bone slices and cultured for 19 hours in bicarbonate buffered control medium, after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.4

A: TOP PANEL

The effect of LTB₄ on TRAP-positive multinucleated cell number. Results are expressed as mean number of TRAP+ MNC per bone slice +/- sem of 16 bone slices for 3 experiments.

* = significantly different from control (p<0.05), using Student’s t-test

B: MIDDLE PANEL

The effect of LTB₄ on the number of lacunae. Results are expressed as mean number of pits per bone slice +/- sem of 16 bone slices from 3 experiments.

C: BOTTOM PANEL

The effect of LTB₄ on osteoclastic bone resorption. Results are expressed as mean area resorbed per bone slice +/- sem of 16 bone slices from 3 experiments.

* = significantly different from controls (p<0.05), using Student’s t-test
V O 1 0 0 1

CONCENTRATION OF LTB (nM)

NUMBER OF TRAP + MNC

NUMBER OF PITS

AREA OF BONE RESORBED (nm²)

CONCENTRATION OF LTB (nM)
Figure 3.4  *The Effect of LTB₄ on Osteoclast Activity*

Osteoclast containing cell populations were plated on bone slices and cultured for 19 hours in bicarbonate buffered control medium, after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.4.

A:TOP PANEL

The effect of LTB₄ on the activity of individual TRAP+MNC Cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of 16 bone slices from 3 experiments.

B:BOTTOM PANEL

The effect of LTB₄ on the size of individual lacunae. Results are expressed as mean of area of bone resorbed per pit +/- sem of 16 bone slices from 3 experiments.
Figure 3.5  *The Effect of LTB₄ on Morphology of Osteoclast*

Representative pictures of osteoclast containing cell populations plated on bone slices at the end of the culture period. Osteoclast containing cell populations were plated on bone slices and cultured for 19 hours in bicarbonate buffered control medium, after which LTB₄ was added for an additional 48 hours of culture. pH of cultures was 7.4. Tartrate Resistant Acid Phophatase positive osteoclasts stain ruby red. Microscopical pictures at 300X magnification.

A:TOP PANEL  Control:

B:BOTTOM PANEL  1000nM LTB₄
Figure 3.6 *The Effect of LTB₄ on Morphology of Resorption Lacunae*

Representative picture of lacunae formed by osteoclast containing cell populations plated on bone slices for 19 hours in bicarbonate buffered control medium after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.4. Osteoclasts stained for Tartrate Resistant Acid Phosphatase are ruby red. Microscopical pictures at 300X magnification.

A:TOP PANEL Control: B:BOTTOM PANEL 1000nM LTB₄
Figure 3.7  *The Effect of LTB₄ on Morphology of Resorption Lacunae*

Representative pictures of resorption lacunae formed by osteoclast containing cell populations plated on bone slices for 19 hours in bicarbonate buffered control medium after which LTB₄ was added for an additional 48 hours of cultures. pH of cultures was 7.4. Resorption Lacunae are stained dark brown. Microscopical pictures at 300X magnification.
Figure 3.8 *The Effect of LTB4 on Osteoclast Numbers and Osteoclastic Bone Resorption in Cultures Maintained at pH 7.0*

Osteoclast containing cell populations were plated on bone slices for 19 hours in HEPES buffered control medium, after which LTB4 was added for an additional 48 hours of culture. The pH of the cultures was 7.0. * = significantly different from control (p<0.05), using Student’s t-test.

A: TOP PANEL

The effect of LTB4 on the number of TRAP-positive multinucleated cells. Results are expressed as mean number of TRAP+ MNC per bone slice +/- sem of 20 bone slices from 2 experiments.

B: MIDDLE PANEL

The effect of LTB4 on the number of lacunae. Results are expressed as mean number of pits per bone slice +/- sem of 16 bone slices from 2 experiments.

C: BOTTOM PANEL

The effect of LTB4 on the total area of bone resorbed. Results are expressed as mean area resorbed per bone slice +/- sem of 16 bone slices from 2 experiments.
Figure 3.9  *The Effect of LTB₄ on Osteoclast Activity*

Osteoclast containing cell populations were plated on bone slices for 19 hours in HEPES buffered control medium after which LTB₄ was added for an additional 48 hours of culture. The pH of the cultures was 7.0. *=significantly different from controls (p<0.05), using Student’s t-test.

**A:TOP PANEL**

The effect of LTB₄ on the activity of individual TRAP+MNC Cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of 16 bone slices from 2 experiments.

**B:BOTTOM PANEL**

The effect of LTB₄ on the size of individual lacunae. Results are expressed as mean of area of bone resorbed per pit +/- sem of 16 bone slices from 2 experiments.
CONCENTRATION OF LTB4 (nM)

AREA RESORBED per TRAP+ MNC (μm²)

MEAN PIT AREA (μm²)

0 100 1000
3.3.2 Effects of 24 and 48 hour incubation periods with LTB₄

In the next series of experiments we evaluated the culture time with LTB₄ required to have an effect. As reported above, 48 hour treatment with LTB₄ significantly increased the number of TRAP+MNC (Figure 3.10.A) and resorptive activity (Figure 3.10.C). At 24 hours, 1000nM LTB₄ treatment had no effect on any of the parameters measured (Figure 3.10 & Figure 3.11). Furthermore, the number of non-osteoclastic cells per microscopic field tripled in between 24 hours and 48 hours in culture with LTB₄.
Figure 3.10  *Osteoclast Numbers and Osteoclastic Bone resorption in Osteoclasts cultured for 24H and 48 H with LTB₄*

Osteoclast containing cell populations were plated on bone slices for 19 hours in bicarbonate buffered control medium after which LTB₄ was added for an additional 24 or 48 hours of culture. The pH of the cultures was 7.4.

A:TOP PANEL  The effect of LTB₄ on the number of TRAP-positive multinucleated cells. Results are expressed as mean number of TRAP+MNC per bone slice +/- sem of 20 bone slices for 3 experiments.

* = significantly different from control (p<0.05), using Student’s t-test

B:MIDDLE PANEL  The effect of LTB₄ on numbers of lacunae. Results are expressed as mean number of pits per bone slice +/- sem of 12 bone slices from 3 experiments.

C:BOTTOM PANEL  The effect of LTB₄ total area of bone resorbed. Results are expressed as mean area resorbed per bone slice +/- sem of 12 bone slices from 3 experiments.

* = significantly different from controls (p<0.05), using Student’s t-test
TOTAL AREA OF BONE RESORBED

CONCENTRATION OF LTB4 (nM)

NUMBER OF PITS

NUMBER OF TRAP+ MNC

48 HOURS IN CULTURE

24 HOURS IN CULTURE
Figure 3.11 *The Effect of LTB₄ on Osteoclast Activity*

Osteoclast containing cell populations were plated on bone slices for 19 hours in bicarbonate buffered control medium after which LTB₄ was added for an additional 24 or 48 hours of culture. The pH of the cultures was 7.4

A: TOP PANEL

The effect of LTB₄ on the activity of individual TRAP+MNC cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of 12 bone slices from 3 experiments.

B: BOTTOM PANEL

The effect of LTB₄ on the size of individual lacunae. Results are expressed as mean of area of bone resorbed per pit +/- sem of 12 bone slices.
3.3.3 Effect of LTB₄ on numbers of TRAP+MNC and on bone resorption in co-cultures of osteoclasts and UMR 106-01 cells.

The observation that our standard osteoclast-containing cultures only responded to LTB₄ after a 48 hour culture period with LTB₄ led us to consider the possibility that the effects of LTB₄ on osteoclasts were mediated through an effect on non-osteoclastic cells. We analyzed this by co-culturing osteoclasts with osteoblast-like cells (UMR 106-01) and testing the effects of LTB₄ in such co-cultures.

A 24 hour co-culture with UMR 106-01 cells significantly increase the number of pits and the total area resorbed compared to osteoclast only cultures (Figure 3.12). 1000nM LTB₄ significantly stimulated bone resorption, increased formation of TRAP-positive multinucleated cells and number of pits within 24 hours of culture in the UMR co-cultures (when compared with control UMR co-cultures). 24 hour treatment with LTB₄ of osteoclasts in the absence of UMR cells had no effect on osteoclastic bone resorption, numbers of TRAP+MNCs or any of the parameters measured.
Figure 3.12  The Effect of a 24 Incubation with LTB₄ on Numbers of Multinucleated Osteoclasts and Area Resorbed in Co-Cultures of Osteoclasts and UMR cells.

Osteoclasts and osteoblast-like cells (UMR 106-01) were co-cultured for 24 hours on bone slices in bicarbonate buffered control medium. The medium was then replaced with medium supplemented with LTB₄ and cultured for a further 24 hours.

A: TOP PANEL
The effect of LTB₄ on the numbers of TRAP-positive Multinucleated Cells in cocultures: LTB₄ (1000nM) had no effect in osteoclast cultures alone. In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased the numbers of multinucleated osteoclasts. Results represent mean number of TRAP+MNC per bone slice +/- sem of 30 bone slices from 3 experiments.

* = significantly different from (OC+UMR) (p<0.05), using Student’s t-test

B: MIDDLE PANEL
The effect of LTB₄ on Numbers of Pits in cocultures: LTB₄ (1000nM) had no effect in osteoclast cultures alone. In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased the numbers of pits. Results represent mean number of TRAP+MNC per bone slice +/- sem of 22 bone slices from 3 experiments.

* = significantly different from (OC+UMR) (p<0.05), using Student’s t-test
# = significantly different from (OC) (p<0.05), using Student’s t-test

C: BOTTOM PANEL
The effect of LTB₄ on total area of bone resorbed in co-cultures: LTB₄ (1000nM) had no effect in osteoclast cultures alone. In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased the numbers of pits. Results represent mean number of TRAP+MNC per bone slice +/- sem of 22 bone slices from 3 experiments.

* = significantly different from (OC+UMR) (p<0.05), using Student’s t-test
# = significantly different from (OC) (p<0.05), using Student’s t-test
TOTAL AREA OF BONE RESORBED (μm²)  

NUMBER OF PITS  

NUMBERS OF TRAP+ MNC  

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>OC</th>
<th>OC + LTB4</th>
<th>OC + UMR</th>
<th>OC + UMR + LTB4</th>
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<td>OC</td>
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<tr>
<td>OC + LTB4</td>
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<tr>
<td>OC + UMR</td>
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<tr>
<td>OC + UMR + LTB4</td>
<td></td>
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</tbody>
</table>
Figure 3.13  
*The effect of LTB$_4$ on Activity of Individual TRAP+MNC cells and Lacunae in Co-Cultures of osteoclasts and UMR cells*

A: TOP PANEL
The effect of LTB$_4$ on activity of individual TRAP+MNC Cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of 22 bone slices from 3 experiments.

B: BOTTOM PANEL
The effect of LTB$_4$ on size of individual lacunae. Results are expressed as mean of area of bone resorbed per pit +/- sem of 22 bone slices.
3.3.5  **Effect of LTB₄ pretreatment of UMR cells on numbers of TRAP+MNCs and on Bone Resorption in co-cultures of osteoclasts and UMR cells.**

To further test the effects of LTB₄ on UMR-mediated osteoclastic activity, we analyzed the effect of pretreating the UMR cells with LTB₄. As shown in Figure 3.14 & Figure 3.15, a 48 hour co-culture with UMR cells induced a significant increase in the number of pits and total area resorbed compared to osteoclast-only controls. Co-culture of osteoclasts with LTB₄ pretreated UMR cells resulted in significantly increased numbers of osteoclasts, numbers of pits and area resorbed compared to co-cultures of osteoclasts and control UMR cells.
Figure 3.14  *The Effect of LTB₄ pretreatment of UMR cells on Numbers of Multinucleated Osteoclasts and Area Resorbed in Co-Cultures of Osteoclasts and UMR cells*

UMR (106-01) cells were cultured on bone slices and pretreated with LTB₄ for 24 hours before co-culture with osteoclasts for an additional 48 hours in bicarbonate buffered control medium at pH 7.4.

A: TOP PANEL

The effect on the Numbers of TRAP+Multinucleated cells: In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased the numbers of multinucleated osteoclasts compared to co-cultures alone (control). Results represent mean number of TRAP+MNC per bone slice +/- sem of 30 bone slices from 3 experiments.

* = significantly different from UMR+OC, (p<0.05) using Student’s t-test.

B: MIDDLE PANEL

The effect on numbers of pits: In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased the numbers of pits compared to cocultures alone (control). Results represent mean number of TRAP+MNC per bone slice +/- sem of 22 bone slices from 3 experiments.

* = significantly different from UMR+OC, (p<0.05) using Student’s t-test.

# = significantly different from OC, (p<0.05) using Student’s t-test

C: BOTTOM PANEL

The effect on total area of bone resorbed: In co-cultures of osteoclasts and UMR cells, LTB₄ (1000nM) increased area of bone resorbed. Results represent mean number of TRAP+MNC per bone slice +/- sem of 22 bone slices from 3 experiments.

* = significantly different from UMR+OC, (p<0.05) using Student’s t-test.

# = significantly different from OCm (p<0.05) using Student’s t-test
Figure 3.15  *The Effect of LTB₄ pretreatment of UMR cells on Activity of Individual TRAP+MNC cells and Lacunae in Co-Cultures of osteoclasts and UMR cells*

A: TOP PANEL
The effect of LTB₄ on activity of individual TRAP+MNC Cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of 16 bone slices from 3 experiments.

B: BOTTOM PANEL
The effect on size of individual lacunae. Results are expressed as mean of area of bone resorbed per pit +/- sem of 16 bone slices.
3.3.6 The Effect of LTB$_4$ on UMR proliferation.

LTB$_4$ at 1000nM concentration had no significant effect on UMR 106-01 proliferation under the culture conditions tested (Figure 3.16). Furthermore, alkaline phosphatase staining showed no differences between treated and control cells (pictures not shown).
Cells were subcultured onto 35 mm² Falcon tissue culture dishes (3 x 10⁴ cells/cm²) and incubated for 24 hours. The cells were then treated with LTB₄. Cell numbers were determined from days one to four of culture by trypsinizing the cells and counting the cell suspension using a Coulter-Counter. Results are expressed as mean number of UMR cells per dish +/- sem of 9 dishes from 3 experiments.
TREATED WITH LTB4

CONTROL
3.4 DISCUSSION

The results described in this chapter show that 48 hours of culture with LTB₄ at higher concentrations (100nM and 1000nM) stimulated bone resorption by increasing osteoclast formation. We also found that a 24 hour incubation with LTB₄ had no effect. Available data suggest that the concentrations used (100nM and 1000nM) are relatively high, but are in accordance with the concentration ranges at which biological activity found by other investigators (Gallwitz et al., 1993, Sandy et al., 1989).

Microscopical observations of osteoclast cultures stained for TRAP suggested that cultures treated with LTB₄ possessed larger osteoclasts with an increased number of nuclei. This is interesting in view of our finding that LTB₄ also increased numbers of TRAP+ multinucleated cells. Resorption lacunae of control bone slices were small while LTB₄ treated bone contained composite type pits in addition to small pits. These results suggest that LTB₄ - stimulation of bone resorption may be partially due to increased numbers of large osteoclasts. The morphology of resorption lacunae clearly suggests that LTB₄ has an affect on the size of the resorption pits. However, calculations of area resorbed per TRAP+MNC or mean pit area show no statistical significance. Lack of statistical significance is related to 1) calculations of area resorbed per TRAP-positive multinucleated cell and mean pit area are derived parameters that include large standard deviations, and 2) that large standard deviations truly reflect the variability that exists in our cultures. In a recent publication, published after we completed our studies, Garcia et al. (1996) similarly reported that LTB₄ caused a dose-dependant increase in numbers of TRAP-positive multinucleated cells in rat osteoclast cultures.

We also demonstrated that LTB₄ - induced increase in the numbers of TRAP-positive multinucleated cells, of area resorbed per bone slice and of the number of resorption lacunae was greater in culture maintained at pH 7.0 than those maintained at pH 7.4. These responses are
consistent with potentiation of osteoclastic resorption in a more acidic pH environment (Shibutani & Heersche, 1993, Arnett & Dempster, 1986).

Several prior studies of the effects of LTB₄ on bone cells have been reported. Meghji and coworkers (1988) were the first to suggest that LTB₄ had any effect on bone cells. They demonstrated that LTB₄ had biphasic effects on bone resorption in calvarial cultures. Bone resorption was stimulated at low concentrations while at higher concentrations, resorption was inhibited. Other investigators have not been able to repeat these results. Furthermore, effects of such low concentrations have not been observed in other systems (Goldman & Goetzl, 1982, Clancy et al., 1987, Powell et al., 1993). It is possible that these previous studies were not conclusive because of the instability of these compounds. Leukotrienes are known to be rapidly inactivated by hepatocytes, monocytes and leukocytes (Keppler et al., 1992) which are commonly found in osteoclast-containing cell populations. Gallwitz and co-workers (1993) showed that the 5-LO metabolites 5-HETE and the peptido-leukotrienes stimulated osteoclastic resorption in cells isolated from osteoclastomas. However, cells isolated from osteoclastomas possess many osteoclast attributes but may respond differently from osteoclasts isolated from normal bone. Recently, Garcia et al. (1996) showed that LTB₄ increased the osteoclastic bone resorption in vivo following local administration over the calvariae of normal mice and in vitro in organ cultures of neonatal mouse calvariae. They also demonstrated that LTB₄ increased the formation of resorption lacunae by isolated neonatal rat osteoclasts.

Garcia et al. (1996) showed that LTB₄ had a biphasic effect in isolated rat osteoclasts with optimal concentration at 10nM in isolated rat osteoclasts. Species differences may be a factor in explaining the differences between this observation and our findings: osteoclasts isolated from newborn rabbits survive for several days in bicarbonate buffered culture medium with 10% FCS, while the osteoclasts isolated from newborn rats survive less than 24 hours under similar culture
conditions (Nicholson et al., 1986). Also, the time required for significance to be obtained is 20 hours in rat osteoclasts while in our experiments at least 48 hours from the beginning of culture was needed. The longer time required for an effect to be realized in rabbit osteoclasts represents some unique problems. Leukotrienes are difficult to quantitate due to 1) short half-life in most biological fluids, 2) their susceptibility to oxidative degradation during sample preparation and 3) albumin binding. It is difficult to ascertain whether the concentrations we used in our experiments were maintained throughout the culture period. We observed in our cultures that the numbers of non-osteoclastic stromal cells increased considerably between 24 h and 48 hr of culture. These observations are interesting in view of the findings of Fenton et al. (1993) who found a 3 fold increase in osteoblast-like cells from 24 h to 48 h of culture in rat osteoclast cultures. They found that the osteoclasts were not responsive to PTH in the first 24 hours but became responsive thereafter. Several in vitro systems have provided strong evidence that accessory cells are necessary to support osteoclast formation in co-cultures with hemopoietic cells (Udagawa et al., 1989, Yamashita et al., 1990). Another possibility is "stromal (induced) osteoclast forming activity" as termed by Chambers et al. (1993). Results obtained by Gallwitz et al. (1993) suggest that 5-LO metabolites may be involved in the communication between accessory cells and osteoclasts.

To test the possibility that the effects of LTB₄ on osteoclasts in our cultures might be mediated through an effect on non-osteoclastic cells, we evaluated the effects of 24 hour LTB₄ treatment in co-cultures of osteoclasts with UMR cells and found that the stimulatory effect of LTB₄ on bone resorption, the number of osteoclasts and pit formation was increased and occurred earlier. Our observations represent one more example of indirect stimulation of osteoclastic activity. To date, there is no direct evidence of LTB₄ receptors in osteoclasts or in osteoblastic cells. However, it is now well established that the classical bone resorption-stimulating
hormones, PTH and 1,25 di-hydroxyvitamin D₃ and several cytokines act indirectly via osteoblasts to activate osteoclasts (McSheeny & Chambers, 1986, 1987, Thomson et al., 1986, 1987, Nicholson et al., 1989 and Hill et al., 1995). Recently, 5-LO deficient mice have been developed by targeted disruption of the 5-LO gene (Chen et al., 1994). These mice may be useful for determining the role of leukotrienes in models of inflammation, and roles associated with osteoclastic bone resorption with inflammatory stimuli and cytokines.

The addition of LTB₄ had no effects on UMR cell proliferation. Ren & Dziak (1991) reported that LTB₄ was able to modulate osteoblastic cell proliferation at concentrations used in our experiments. They found that LTB₄ inhibited proliferation in normal osteoblasts derived from rat calvaria and that LTB₄ stimulated Saos-2 and G292 clonal cell lines. The differences may be related to the particular clonal cell lines used, which may represent cells in different stages of osteoblast differentiation and may therefore respond differently.

The strongest evidence that stimulation of bone resorption by LTB₄ is mediated by osteoblast-like cells is provided by the experiments in which UMR 106-01 cells pretreated with LTB₄ stimulated bone resorption and increased numbers of osteoclasts and numbers of resorption lacunae. Interestingly, in all of the osteoblast pretreatment experiments, the magnitude of LTB₄-induced stimulation ranged from 1.30-1.79 times the control values and, was less than that seen in co-culture experiments in which both cell populations were exposed simultaneously to the cytokine. This apparent reduction in response may be due to the contribution of direct exposure of the stromal cells to LTB₄, to lability of an osteoblast-derived factor or message that is responsible for osteoclast stimulation or to an additional effect of direct exposure of osteoclasts to LTB₄.

In similar experiments with thyroid hormones and parathyroid hormone, Britto et al. (1994) also found that these hormones produced greater stimulation of bone resorption in
osteoblast-osteoclast co-cultures in which both populations were exposed to PTH, than in experiments in which osteoblastic cells were pre-treated with the hormone before co-culture.

In all our co-culture experiments, LTB₄ did not alter the mean area resorbed per osteoclast or pit size, thus the increased bone resorption may have been due to an increase in the number of resorption events undertaken by a single osteoclast after LTB₄ addition. Alternatively, the proportion of osteoclasts activated may be increased in the presence of LTB₄ suggested by increased formation of resorption lacunae.

In summary we have shown that LTB₄ is a potent stimulator of osteoclastic bone resorption and a powerful stimulator of osteoclast formation. Our data also indicates that LTB₄ stimulates osteoclastic bone resorption by acting on osteoblast-like cells and stromal cells.
CHAPTER 4

THE EFFECT OF THE LTB₄ ANTAGONIST LY 292 728

ON THE DEVELOPMENT OF TRAP + CELLS,

NUMBERS OF RESORPTION LACUNAE AND BONE RESORPTION

IN ISOLATED OSTEOCLAST CULTURES
4.1 INTRODUCTION

In this chapter we have investigated the role of LTB₄ in osteoclast formation and bone resorption through the use of a specific receptor antagonist, LY 292 728. We evaluated the effect of blockade of endogenous LTB₄ and blockade of exogenously added LTB₄ on development of TRAP+MNC, on the numbers of resorption lacunae and the effects of bone resorption using isolated osteoclasts cultured on bone slices.

Since the elucidation of the 5-LO biosynthetic pathway, there has been much debate whether inhibition of the 5-LO enzyme or using specific antagonists is the best approach. 5-LO inhibitors block the formation of the full spectrum of 5-LO products, while a leukotriene antagonist blocks the effect of only one leukotriene. LY 292 728 (LY hereafter, developed by Eli Lilly) is a newly synthesized potent LTB₄ antagonist (Ki of 0.5nM) whose specificity for the receptor (LTB₄ Kd of 1.9nM) ranks amongst the highest available (Sawyer et al., 1995).
4.2 MATERIALS AND METHODS

4.2.1 Rabbit Osteoclast Isolation and Culture Conditions

Rabbit osteoclasts were isolated as described in Chapter two. Based on the results presented in chapter 2, the culture conditions chosen were the following:

Neonatal rabbit osteoclasts were prepared and plated on either 35 mm tissue culture dishes or on 6mm devitalized cortical bone slices in a 96 well microtiter plates in 2% medium.

35 mm Tissue Culture Dish: The cells were plated on culture dishes and allowed ninety minutes for attachment before the addition of 2 ml α-MEM with 2% FCS and antibiotics. After 24 hours of culture, cells were washed to remove non-adherent cells and red blood cells. The medium was then replaced with control medium or medium containing 1000nM LY in the concentration indicated and cultures continued for an additional 48 hours.

Devitalized Cortical Bone Slices: Bone slices were placed in the wells of 96 well microtiter plates and 150uL of medium added. Then 50 ul of cell suspension was plated onto the 150uL of medium already in the well. There were three experimental groups: 1) After 24 hours in culture, medium was removed and replaced with fresh medium containing with or without LTB₄ and cultures continued for 48 h. 2) After 24 hours in culture, medium was removed and replaced with fresh medium containing both LY and LTB₄ and the cultures continued for an additional 48 hours. 3) After 6 hours in culture, medium was removed and replaced with fresh medium with or without LY and cultures continued for 18 hours. The medium was then replaced with medium containing LY and LTB₄ and culture continued for an additional 48 hours. All cells were cultured at 37 C in a humidified atmosphere of 95% air and 5% CO₂ in bicarbonate buffered medium at pH 7.4.
4.2.2 Evaluation of Cultures

At the end of the culture period the cultures were fixed and stained for TRAP. Cells containing two or more nuclei were classified as TRAP positive multinucleated cells (TRAP+MNC).

4.2.3 Quantitation of Bone Resorption and Number of Lacunae

After enumerating TRAP+ cells, bone slices were immunostained for resorption lacunae (described in section 3.2.3) and quantitated as described in section 3.2.4.

4.2.4 Statistical analysis

Experiments using culture dishes consisted of groups n>=6, and experiments conducted on bone were done twice with n>=8 bone slices. Statistical analysis was performed for each experiment by two tailed unpaired Student’s t-test.
4.3 RESULTS

4.3.1 Effects of LTB₄ antagonist LY 292 728 on the numbers of TRAP+MNC and Bone Resorption at pH 7.4

We first tested the effects of LY on osteoclast formation in rabbit osteoclast-containing cultures on plastic substratum. The results shown in Figure 4.1 illustrate that treatment with 1 - 1000nM of antagonist for 48 hours had no effect on the number of TRAP-positive multinucleated cells. Furthermore, no cytotoxic effect based on numbers of TRAP-positive cells nor any change in osteoclast morphology was observed. Given that no toxic effects were observed, we selected a concentration of 1000nM LY for our antagonist studies.

We next examined the effect of 1000nM LY on resorptive activity and formation of osteoclasts in cultures on bone slices. After 48 hours of culture in the presence of LY, no significant effects were detected on any of the parameters measured (Figure 4.2). Simultaneous treatment of osteoclasts with LY and LTB₄ inhibited bone resorption and pit formation induced by 100nM LTB₄, but not by 1000nM LTB₄. Pretreatment of osteoclasts with LY followed by simultaneous addition of LY and LTB₄, appeared to be slightly more effective in decreasing numbers of TRAP+MNCs, formation of pits and bone resorption when compared to the LY and LTB₄ treated groups. With respect to all antagonist treated groups, mean area resorbed per osteoclast and pit size was not significantly altered by LY treatment.
Figure 4.1 The effect of LY 292 728 on formation of TRAP-positive multinucleated cells

Osteoclast containing populations were plated in 35 mm culture dishes and cultured for 19.5 hours in bicarbonate buffered control medium, after which 1000nM LY was added for an additional 48 hours of culture. The pH of cultures was 7.4.

Results are expressed as mean number of TRAP+MNC per dish +/- sem of n=6 dishes.

* = significantly different from control (p<0.05)
Figure 4.2  **The Effect of LY 292 728 on Osteoclast Numbers, Osteoclastic Bone Resorption and Osteoclastic Activity.**

Osteoclast containing cell suspension in bicarbonate buffered control medium was plated on bone slices. Three treatment regimens were used.  
1) After 24 hours in culture, medium was removed and replaced with fresh medium with or without LTB₄ and cultures were continued for 48 H. 
2) After 24 hours in culture, medium was removed and replaced with fresh medium with LY and LTB₄ simultaneously and the cultures continued for an additional 48 hours.  
3) After 6 hours in culture, medium was removed and replaced with medium containing LY and culture continued for 18 hours, after which the medium was replaced with medium containing both LY and LTB₄ and culture continued for an additional 48 hours. The pH of the cultures was 7.4

**Figure 4.2.A** The effect of LY 292 728 on the number of TRAP-positive multinucleated cells. Results are expressed as mean number of TRAP+MNC per bone slice +/- sem of n>=8 bone slices from 2 experiments.  
- # = significantly different from control (p<0.05)  
- * = significantly different from 100nM LTB₄ (p<0.05)  
- + = significantly different from 1000nM LTB₄ (p<0.05)

**Figure 4.2.B** The effect of LY 292 728 on Numbers of Lacunae. Results are expressed as mean number of pits per bone slice +/- sem of n>=8 bone slices from 2 experiments.  
- # = significantly different from control (p<0.05)  
- * = significantly different from 100nM LTB₄ (p<0.05)  
- + = significantly different from 1000nM LTB₄ (p<0.05)

**Figure 4.2.C** The effect of LY 292 728 on total bone resorbed. Results are expressed as mean area of bone resorbed per bone slice +/- sem of n>=8 bone slices from 2 experiments.  
- # = significantly different from control (p<0.05)  
- * = significantly different from 100nM LTB₄ (p<0.05)  
- + = significantly different from 1000nM LTB₄ (p<0.05)

**Figure 4.2.D** The effect of LY 292 728 on Activity of Individual TRAP+MNC cells. Results are expressed as mean area of bone resorbed per TRAP+MNC +/- sem of n>=8 bone slices from 2 experiments.

**Figure 4.2.E** The effect of LY 292 728 on Size of Individual Lacunae. Results are expressed as mean area of bone resorbed per bone slice +/- sem of n>=8 bone slices from 2 experiments.
E

- LTB4 only
- CONTROL
- 1000nM LY 292 728 pretreatment, followed by LTB4 simultaneous treatment
- LY only
- 1000nM LY 292 728 and LTB4 simultaneous treatment

**MEAN PIT AREA (µm²)**

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>MEAN PIT AREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>2000</td>
</tr>
<tr>
<td>1000 nM LY</td>
<td>1500</td>
</tr>
<tr>
<td>100 nM LTB4</td>
<td>1800</td>
</tr>
<tr>
<td>1000 nM LTB4</td>
<td>2200</td>
</tr>
<tr>
<td>1000 nM LY 292 728</td>
<td>2100</td>
</tr>
</tbody>
</table>
4.4 Discussion

LY at 1-1000nM had no effect on formation of TRAP-positive multinucleated cells or on osteoclastic bone resorption in control cultures. We concluded from this that endogenous LTB$_4$ production is not involved in osteoclast formation in control cultures.

LY pretreatment of osteoclasts, followed by simultaneous addition of LY and LTB$_4$ was more effective at decreasing numbers of TRAP-positive multinucleated cells, formation of resorption pits and bone resorption than simultaneous treatment with LY and LTB$_4$. A possible explanation could be that although LY 292 728 is a reversible competitive antagonist, prior binding to the receptor may decrease the ability of LTB$_4$ to bind to the receptor. When cells are treated with the agonist and antagonist together, the compounds are competing for the same sites, which may decrease the efficiency of the antagonist blockade. This was also the case in some of the other antagonists developed by Eli Lilly in the LY 292-series of compounds but no studies are available on LY 292 728 (personal communication, Dr. Jackson). Franchi-Miller & Saffer (1995) tested the effects of an inhibitor of 5-LO on bone remodeling and found that numbers of osteoclasts significantly decreased. Flynn et al. (1995) also found that the use of an 5-LO inhibited blocked the 1,25 di-hydroxyvitamin D$_3$ induced stimulation of multinucleated cell formation only when added at the beginning of culture but not later during the culture period.

Microscopical analysis of osteoclasts stained for TRAP gives the impression that antagonist treatment in the presence of LTB$_4$ blocked the formation of larger osteoclasts with multiple nuclei otherwise found in LTB$_4$ treated groups. In addition, we observed differences in lacunar morphology. Interestingly, when comparing morphology of antagonist treated cells, (1000nM +100nM, 1000nM LY + 1000nM LTB$_4$ at beginning of cultures and 1000nM LY + 100 LTB$_4$ during cultures) we found an absence of large composite pits and a tendency towards
decreased pit size and decreased area resorbed per TRAP+MNC cells. However, in the 1000nM LY and 1000nM LTB₄ - treated groups there was a reappearance of composite pits. Surprisingly, with the 100nM LTB₄ +1000nM LY treatment, there was a decrease in number of pits formed but no significant decrease in TRAP+MNCs.

In summary, we have shown that LY, a potent LTB₄ receptor antagonist, inhibited LTB₄ induced TRAP+MNC formation, pit formation and osteoclastic bone resorption. This confirms and extends results shown before that LTB₄ is involved in the recruitment or differentiation of mononuclear preosteoclasts into multinucleated osteoclasts.
CHAPTER 5

SUMMARY AND CONCLUSIONS
5.1 SUMMARY & CONCLUSION

Bone resorption, the physiological process by which the mineral phase of bone is solubilized, is important for the maintenance of plasma calcium levels and for the normal remodelling of bone in response to altered patterns of mechanical loading. Bone resorption is the result of two mechanisms, one being the generation of new osteoclasts and the other the activation of existing osteoclasts. Bone resorption requires the cooperation between osteoclasts and mononuclear accessory cells by mechanisms which are unknown.

There are previous reports that leukotrienes may be modulators of bone cells. Meghji et al. (1988) investigated purified leukotrienes in mouse calvarial assay and found significant bone resorption. Gallwitz et al. (1993) tested the effects of 5-LO pathway metabolites in osteoclastoma cultures, and found that 5-LO metabolites stimulated osteoclastic resorption. Since leukotriene B₄ is one of a number of agents known to be present at sites of inflammation associated with bone loss and given the strong evidence by Gallwitz et al. (1993) that another 5-LO pathway metabolite, may be involved in the regulation of osteoclasts, we investigated the role of LTB₄ on the physiological regulation of bone remodelling. In a recent publication, Garcia et al. (1996) showed that LTB₄ stimulated a dose-dependant formation of osteoclasts and osteoclastic bone resorption in rat osteoclasts.

In the first series of experiments, we established culture conditions which we believed were essential to be able to study LTB₄ - mediated regulation of numbers and activity of osteoclasts. Use of 2% serum concentrations resulted in adequate osteoclast numbers and or survival and minimized proliferation of stromal cells.

In the second series of experiments, we found that LTB₄ - treatment increased osteoclast numbers and that this resulted in increased osteoclastic resorption by increasing the number of active osteoclasts. Lowering the pH of the medium potentiated osteoclastic bone resorption,
and the effects of LTB₄ appeared to be more pronounced at this pH. Since inflammatory conditions are associated with a low pH environment, this may also contribute in vivo situations.

An interesting finding in these experiments was the appearance of larger multinucleated cells after LTB₄ treatment. Whether multinuclearity confers any special ability on the osteoclast, such as is reported for macrophages (Vignery et al., 1988) is unknown. Piper et al. (1992) observed a positive relation between number of nuclei per osteoclast and the volume of the pit made. This may explain the larger composite pits found in LTB₄ treatment compared to control.

We found that a 24 hour incubation with LTB₄ had no effect, while after 48 hours, LTB₄ increased bone resorption and the number of osteoclasts. Since in these experiments, the number of TRAP+MNCs and stromal cells increased between 24 hours and 48 hours of culture, we tested the possibility that the effects of LTB₄ on osteoclasts might be mediated through an effect on non-osteoclastic cells. Our finding that treatment for 24 hours with LTB₄ stimulated bone resorption, the number of osteoclasts and pit formation in the presence, but not in the absence of UMR cells is strong evidence that osteoblast-like cells mediated the LTB₄ induced stimulation. Thus the presence of stromal cells and osteoblast lineage cells might provide a microenvironment in which osteoclasts function more efficiently. Agents such as PTH, IL-1, TNF-alpha, lymphotoxin, TGF-beta and prostaglandins, all stimulate osteoblasts to produce a soluble resorption stimulating factor which in turn can induce bone resorptive activity. LTB₄ can now be added to this list. To date, an intense search to identify and purify such a specific osteoclast stimulating factor produced by osteoblast-like or stromal cells has been unsuccessful.

Bone surfaces are covered by an extended cellular membrane of bone-lining osteoblast-like cells. One of the actions of a stimulator of bone resorption may be to make these surfaces available to osteoclasts by inducing the osteoblast layer to retract (Rodan & Martin, 1981). This
hypothesis would reinforce the role of osteoblasts as the initial target cell and mediator of activity (Puza & Ishibe, 1993). This hypothesis is intriguing in view of our impression that co-cultures treated with LTB₄ showed a change in stromal/osteoblast cell distribution. During cultures, osteoblasts were initially spread out evenly across the bone slices, but with LTB₄ addition, the distribution of osteoblasts aggregated into groups exposing what seemed to be more of the bone surface to the osteoclast.

In the last experimental chapter, we explored the role of LTB₄ in osteoclast-containing cultures through the use of a specific LTB₄ receptor antagonist. This antagonist by itself did not affect formation of osteoclasts or osteoclastic bone resorption, providing indirect evidence that endogenous LTB₄ is not normally produced by osteoclast cultures in concentrations that have a biological effect. We then examined the effects of LY on LTB₄ induced effects and demonstrated that LY inhibited the LTB₄ - induced osteoclastic resorption and appearance of numbers of TRAP-positive cells. Greatest differences were demonstrated in osteoclast-containing cell populations which were pretreated with LY before adding LTB₄.

Recently, in vivo studies with LTB₄ have shown that local administration of LTB₄ over the calvaria of mice increased osteoclastic bone resorption, numbers of osteoclasts and activity (Garcia et al., 1996). These findings strongly support our findings in vitro. Furthermore, the concentrations of LTB₄ we found to stimulate osteoclastic resorption are concentrations found in the synovial fluid of patients with rheumatoid arthritis (Rae et al., 1982; Davidson et al., 1983). 5-LO deficient mice have recently been developed by targeted disruption of the 5-LO gene (Chen et al., 1984). These mice are resistant to the lethal effects of shock induced by platelet activity factor, ear inflammation induced to arachidonic acid metabolites and immune complex-induced injury. These mice may be useful for determining the role of leukotrienes in models of inflammation.
In conclusion, we have shown that LTB₄ is a potent stimulator of osteoclastic bone resorption and osteoclast formation. We have also shown that these stimulatory effects are mediated through effects on osteoblast-like cells. This is similar to previous observations for other bone resorbing hormones and cytokines, which also increase osteoclastic activity indirectly, via a primary effect on the osteoblasts.

Future studies should attempt to define the mechanism of action of LTB₄ on osteoblast-mediated activation of osteoclasts. It would be of great interest to identify the cells that possess receptors for LTB₄ and the cells responsible for producing LTB₄. Better understanding of the mechanism and role of LTB₄ may provide a means for treating of several bone diseases characterized by elevated leukotriene levels and is worthy of further investigations.
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